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RANKL AND OSTEOPROTEGERIN LEVELS IN RESPONSE TO ORTHODONTIC FORCES

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ABSTRACT

Orthodontic tooth movement is mediated by interactions between PDL cells and those of the alveolus. One protein—the receptor activator nuclear factor kappa B ligand (RANKL)—is critical for osteoclastogenesis, and osteoprotegerin (OPG) is a decoy ligand that competitively inhibits RANKL. A higher RANKL/OPG ratio is associated with areas of bone resorption, while a lower ratio occurs in areas of bone deposition and homeostasis. There have been almost no clinical studies of RANKL and OPG expression in human subjects undergoing orthodontic tooth movement.

The purpose of this study was to quantify changes in the levels of RANKL and of OPG expression in human gingival crevicular fluid in growing (adolescent) and non-growing (adult) patients in response to orthodontic force. Untreated adolescents (under 18 years of age) and adults (over 18 years of age) had a calibrated force applied across a left-right pair of maxillary premolars with a transpalatal spring (TPS). RANKL and OPG were measured in gingival crevicular fluid (GCF) sampled serially from the pressure and tension sides of maxillary premolars at 5 different time points: before placement of transpalatal spring, 1 day (24 hours) after TPS placement, 2 days (48 hours) after TPS placement, 5 days (120 hours) after TPS placement (TPS was then removed), and 3 days (72 hours) after TPS removal. RANKL and OPG expression was measured by the ELISA assay. Expectations were that (1) force would raise RANKL and diminish OPG, (2) force removal would reverse the RANKL-OPG levels, (3) strength and duration of force are associated with RANKL-OPG levels, (4) responses would exhibit considerable inter-individual variation, (5) the RANKL/OPG levels will be higher in children due to general growth and bone remodeling in response to orthodontic forces, and (6) the RANKL/OPG levels will be lower in adults because no growth is occurring and osteoclastogenesis is triggered solely by orthodontic forces.

Gingival crevicular fluid (GCF) volume increased significantly after applying force with the nickel-titanium coil spring. The volume remained elevated until the force was removed, and had not quite returned to baseline by 3 days (72 hours) following force removal. There were no significant differences found in the levels of GCF collected in regards to age, sex, or race. When evaluating the changes in gingival crevicular fluid in response to mechanical force, it appears that *time* is the significant factor regardless of the person's age, sex, or race. In general, both RANKL and OPG levels decreased significantly over the time while the spring was in place; however, the RANKL/OPG ratio increased over time. No significant differences were found in the levels of RANKL when comparing sex, age, and race. Higher levels of OPG were found in males. A linear decrease in OPG was seen in regards to age; therefore, adults have less OPG than their younger counterparts. Although not significant, African American levels of OPG were higher than Caucasians. Further research is needed to better describe what effect

variations in individual RANKL and OPG expression have on orthodontic tooth movement.

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CHAPTER 1. INTRODUCTION

Bone is described as a simple tissue comprised of four major components namely: cells, the extracellular matrix of collagen fibers, mucopolysaccharide ground substance, and calcium salts. Although bone is a simple material, it is also dynamic and is constantly undergoing remodeling and mineralization. Bone remodeling can be physiological, which is the most common, pathological, or mechanical in origin. For physiological bone remodeling to occur, a balance must be maintained between osteoblastic (synthesis) and osteoclastic (resorption) activities. When this balance is disturbed naturally, pathological bone remodeling will occur, leading to destructive bone arthropies. When orthodontic (mechanical) forces are applied to a tooth, the forces are transmitted through the periodontal ligament (PDL) and adjacent alveolar bone. The transmitted forces ignite a chain of signal transduction events leading to a shift in the osteoblastic/osteoclastic ratio. During orthodontic force application, areas of compression and tension develop. On the side of the tooth where the PDL fibers are stretched (tension), an environment forms where osteoblastic activity prevails and new bone is formed. Areas in which the PDL fibers are under compression exhibit increased osteoclastogenesis, with the end result being bone resorption (Masella and Meister 2006).

The culmination of events leading to orthodontic tooth movement (OTM) is complex, and includes interaction between the alveolar bone cells and PDL cells along with intercellular actions. The sequence of events at the tissue and cellular levels during OTM has been thoroughly documented and is well understood. Yet, there is a lack of knowledge surrounding the culmination of biochemical events at the molecular level in the response to a mechanical or orthodontic force (Krishnan and Davidovitch 2006). Recently, orthodontics has been given a new appreciation for the complexity of the events leading to OTM through the discovery of multiple regulatory molecules and signal transduction pathways.

Research on the molecular biology of bone has produced two osteoblast-derived factors that play key roles in bone growth and remodeling. The receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) competitively bind to their receptor, RANK. These molecules exert counterbalancing regulatory effects on osteoclastogenesis, including osteoclast differentiation, activation, and survival, and are as a result critical for initiation and maintenance of orthodontic tooth movement (Kanzaki *et al.* 2006). The aim of the present study is to measure changes in RANKL and OPG expression in human gingival crevicular fluid in growing (adolescent) and non-growing (adult) patients in response to orthodontic force.

Little is known about the levels of RANKL and OPG in human gingival crevicular fluid (GCF), and most of the studies that have assayed GCF have been

concerned with people with periodontitis and endodontic lesions. The purpose of this study was to evaluate:

1) What are the levels of RANKL and OPG in the gingival crevicular fluid in the absence of orthodontic force? Kawasaki *et al*. (2006) found that the levels of RANKL and OPG increase with age during their cross-sectional evaluation comparing a sample of adolescents and a sample of adults. This study assessed 54 participants of various ages. Although there were no set age groupings for this study, in order to treat age as a category, a number of the patients will be less than 16 years of age or older than 30 years. Genetics appears to play a critical role in a person's susceptibility to external apical root resorption (Hartsfield *et al.* 2004). Therefore, we are also interested in the inter-individual variability of the sample group, since differences in RANKL levels appear to be associated with a person's risk for external apical root resorption (*e.g.*, Harris 1997; Harris *et al*. 2000).

2) Transpalatal springs were placed across a pair of maxillary premolars as the "stressor" used to stimulate RANKL production (and down-regulate OPG production). Several questions were addressed here. One, the mechanical force associated with changes in RANKL-OPG levels from the baseline at T1 (Day 0). Expectation was that RANKL would increase and OPG would drop. Serial sampling at 1 (T2), 2 (T3), and 5 (T4) days will provide information on whether the sustained tension promotes sustained levels of the molecules, or whether the responses are dynamic over time. Two, force-removal right after T4 (Day 5) is used to test the recovery of the system towards or, even, to the baseline levels during the subsequent days (T4 to T5, or Days 5 to 8). It also was of interest whether the duration-to-cytokine levels were simply linear or more complex in nature, and this was assessed by fitting regression models to the data to seek maximum explained following force degradation variance (*e.g.*, Freund and Littell 1991). Nishijima *et al.* (2006) reported that RANKL levels were significantly increased while the OPG levels were significantly decreased at 24 hours after application of a retracting force. The other time points he examined were zero hours, one hour, and 168 hours (7days), which showed no significant difference in the RANKL and OPG levels. Therefore, it can be concluded that RANKL and OPG levels return to normal.

CHAPTER 2. REVIEW OF THE LITERATURE

Temporal Phases of Orthodontic Tooth Movement

The typical tooth movement response after a moderate, continuous load has been divided into three stages. The three temporal phases of tooth movement are (1) the initial strain, (2) the lag phase, and (3) progressive tooth movement (Graber 2005; Reitan 1967). The initial strain occurs between 4 to 7 days after the initiation of mechanical stress. This initial displacement of up to a millimeter is caused by trifold events. First, the root is displaced into the periodontal ligament. Second, the bone is strained caused by bending and creep, and finally, extrusion is caused by the inclined plane effect of the tooth root pressing against the tapering alveolus (Graber 2005). The initial strain response will differ according to the width of the PDL, root length, anatomical configuration, force magnitude, occlusion and periodontal health. Initial tooth displacement occurs instantaneously following mechanical loading; however, the actual compression of the PDL requires an extended duration of force (Graber 2005).

The lag phase, which is variable in length depending on the magnitude of the applied force, consists of undermining resorption. Undermining resorption is a process by which bone adjacent to the areas of crushed PDL is removed (Graber 2005). The lag phase can last from a few days up to 10 weeks depending on the density of the strained bone (Graber 2005; Reitan 1967). During this phase, the cells in the PDL disappear due to a loss of vascular supply, and a hyalinized (cell free) zone in the PDL appears (Proffit 2007; Reitan 1967). Remodeling of bone in areas adjacent to the necrotic tissue is accomplished by cells derived from undamaged areas. After a small delay, cellular elements begin to invade the hyalinized area, and undermining resorption commences. Osteoclasts appear in the adjacent bone marrow spaces and begin to attack the underside of bone immediately adjacent to the necrotic PDL area (Proffit 2007). During this period, an interruption in tooth movement is expected due to (1) a delay in stimulating cellular differentiation within the marrow spaces and (2) a significant amount of bone must be removed from the underside of the hyalinzed zone before tooth movement can occur. Excessive forces lead to larger hyalinzed areas, while light forces minimize but do not eliminate undermining resorption (Graber 2005; Proffit 2007).

The final temporal phase is known as progressive tooth movement, and it is a period of direct bone resorption that allows the tooth to continue to move (Reitan 1967). The rate limiting step in progressive tooth movement is frontal resorption in the PDL, and it determines the speed of orthodontic correction that can be achieved (Graber 2005). Prostaglandin E (PGE) has both osteoclastic and osteoblastic activities; therefore, it is a fitting mediator of tooth movement. Both osteoblasts and osteoclasts are needed for this phase of orthodontic tooth movement. Osteoclasts arrive in areas adjacent to the compressed PDL either from the local cell population or are brought in to the necrotic

area via blood flow. Then, the osteoclasts attack the adjacent lamina dura and remove bone in a process known as frontal resorption. Tooth movement begins shortly after frontal resorption starts. Later, after the PDL is enlarged, osteoblasts, which are recruited from local progenitor cells in the PDL, form bone on the tension side and begin remodeling on the pressure side. See **Figure 1**.

Bone Metabolism and Orthodontic Tooth Movement (OTM)

Bone is a dynamic structure that is constantly undergoing remodeling and mineralization. Bone consists of multiple cell types in an extracellular matrix of collagen fibers, mucopolysaccharide ground substance, and calcium salts (Storey 1972). Goals of bone remodeling include regulation of the body's free calcium ion supply, maintenance of the blood supply to osteocytes, which are embedded in the bone, and adaptation of the bone to the stresses and strains of function. A balance between osteoclastic and osteoblastic actions is absolutely necessary for physiological bone remodeling (Massella and Meister 2006; Storey 1972).

The theories of tooth movement on the both tissue and cellular levels have evolved dramatically over the past century. Recent discoveries deal with the nature of tooth movement on the molecular level. According to Schwarz (1932), the first mention of the biology of tooth movement to appear was in 1904 by Carl Stanstedt. His investigations showed that a tooth tilts along an axis lying slightly apical to the center of the length of the root, which allows the alveolar wall on the side under tension to experience bone deposition, while the side under pressure experiences bone resorption. Oppenheim (1942) published contradictory findings, theorizing that a tooth tilts at its apex, causing transformation over a broad area on both sides of the bony alveolar wall due to both the compression and tension combined. Schwarz (1932) explained the differences between Stanstedt's and Oppenheim's findings by stating that Oppenheim did not see the acute effect of the applied force because he sacrificed his experimental animals several days after the appliance had last been activated.

Storey (1972) discussed three different biologic systems for the movement of teeth. These are the bioelastic phenomena, bioplastic adaptive behavior, and biodisruptive deformation of tissues. Magnitude of force and frequency of application determine whether the tooth moves within its socket or is translated through the bone. The support system of teeth includes the interstitial fluid, the periodontal ligament (PDL), and the viscoelastic properties of the ligament (Henneman *et al.* 2008). Forces exceeding the bioelastic limit result in changes in the connective tissues and vascular system, which leads to adaptive proliferation and remodeling of the tissues. Forces that exceed the bioplastic limit result in biodisruptive deformation, leading to interference in nutrition, ischemia, inflammation, rupture of connective tissues, and localized cell death. Orthodontic forces that are absorbed by the PDL and adjacent alveolar bone are

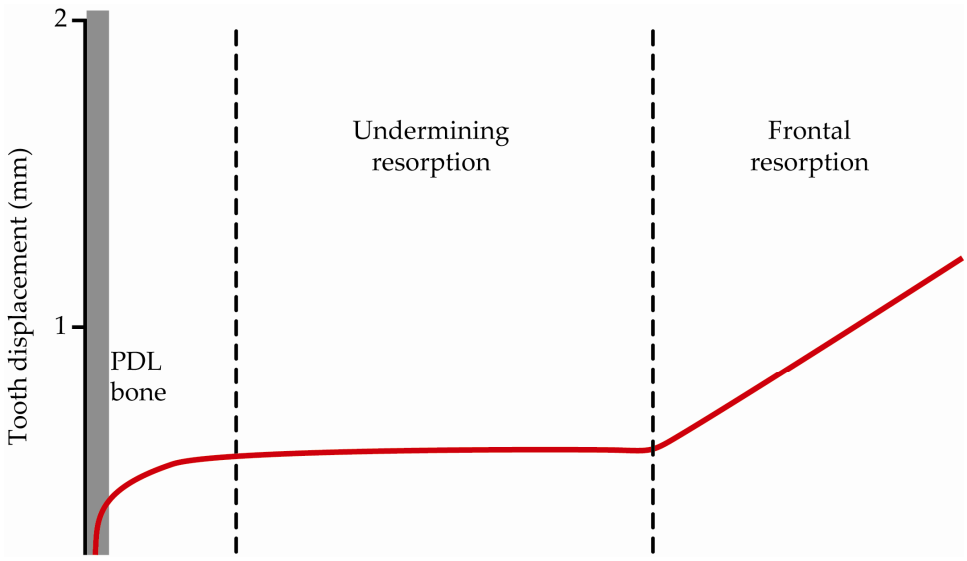

Figure 1. The three phases of tooth movement: (1) initial strain; (2) variable lag phase; and (3) progressive tooth movement.
After application of a moderate orthodontic load (0.2 to 0.5 n, or about 20 to 50 g), tooth displacement can be divided into three phases: (1) initial strain for 1 to 3 days in the periodontal ligament (PDL) and supporting bone; (2) a variable lag phase, in which undermining resorption removes bone adjacent to crushed areas in the PDL; and (3) progressive tooth movement through frontal resorption in the PDL limits the rate of orthodontic tooth correction (Graber 2005).

Source: Reprinted with permission. Graber TM, Vanarsdall RL, Vig KWL, editors. Orthodontics. Current Principles and Techniques, 4th Edition. St Louis: Mosby Elsevier 2005.

therapeutic when they lie between the bioelastic and bioplastic limits. When force exceeds the bioplastic range, tooth movement occurs more slowly. Even heavier forces cause tooth movement to cease due to compression of the connective tissue on the pressure side being compressed into the bone. Regardless of the amount of force, the inflammatory process is limited to the periodontal tissue and does not extend beyond the bone that supports the teeth. The tissue involved in orthodontic tooth movement acts like a viscoelastic material, and consequently strain in the PDL is related to time (*i.e.*, length of force application) and to stress (Storey 1952; Storey 1972).

Application of orthodontic forces on a tooth will cause a shift in osteoblastic and osteoclastic dynamics. On the side of the tooth where the PDL fibers are being stretched (the tension side) an osteoblastic environment is formed, where the osteoblasts enlarge and multiply and new bone is formed. Conversely, on the side under pressure, a process of undermining resorption is initiated due to the tooth compressing the periodontal tissues against the bony socket. The compression side exhibits osteoclastogenesis, which leads to increased activity of osteocytes and bone resorption (Massella and Meister 2006; Storey 1972). Bone resorption does not occur along the pressure side until the cell-free area has been eliminated by undermining resorption. A hyalinized zone is produced by light forces, so the underlying bone is readily removed by resorption (Reitan 1967). Eventually, new bone formation occurs in the direction of the applied force in the form of projecting trabeculae on the periosteal aspect of the alveolar plate (Meikle 2006; Roberts *et al.* 1981; Stanstedt 1904).

As established by Roberts, Goodwin, and Heiner (1981), bone remodeling in response to orthodontic force can be divided into three major categories: (1) turnover, in response to accumulation of microfractures; (2) reorientation of bone mass to optimally resist stress; and (3) net change in volume related to functional load. This can be simplified as an activation, resorption, and formation (ARF) sequence at the tissue level. The critical factors at the cellular level of tooth movement include the distribution of stress, displacement of the PDL, and bone deformation. Cell perturbation, bioelectrical signals, microenvironmental factors and accumulation of microfractures have all been considered as causative to the biophysical events in the cellular response to tooth movement. It has been discovered that mechanical strain activates multiple cell signaling pathways (Meikle 2006). During orthodontic tooth movement, the inflammatory pathway is activated via phospholipase A_2 that causes the release of arachidonic acid followed by the synthesis of prostaglandin E_2 (PGE_2). The increase in PGE_2 activates adentylate cyclase that causes a brief increase in intracellular cAMP. The increased intracellular cAMP then causes an increase in intracellular calcium and the stimulation of DNA synthesis (Meikle 2006). To provide further evidence that prostaglandins are involved in orthodontic tooth movement, the use of cyclooxygenase-1 inhibitors (COX-1) have been linked to decreased tooth movement (Harrell *et al.* 1977; Yamasaki *et al.* 1980). Another metabolite of arachidonic acid is leukotriene, which is

produced by the lipoxygenase pathway, is a potent stimulator of bone resorption (Meikle 2006).

Two other signaling pathways have been identified in the mechanical force transduction. First, mechanical force causes an activation of the cAMP messenger system. cAMP and cGMP are secondary messengers associated with bone remodeling, and they are mediated through the phosphorylation of certain substrate proteins by their dependent protein kinases (Krishnan and Davidovitch 2006). At first, the application of mechanical force causes a decrease in cAMP levels and an increase of calcium uptake into the cells. Shortly thereafter, cAMP levels rise, and bone remodeling activities commence (Meikle 2006). Lastly, the phosphoinositide pathway (PIP) is activated by prostoglandin E_2, and parathyroid hormone (PTH), and the pathways' by-products cause a release of intracellular calcium. This pathway is responsible for the elevation of intracellular calcium via the endoplasmic reticulum and increased DNA synthesis (Krishnan and Davidovitch 2006; Meikle 2006). As technology improved, it was discovered that these signaling pathways lie downstream of the initial mechanoreceptor event, which causes the stimulation of specialized proteins, known as cytokines, to initiate the process of bone remodeling.

Molecular Elements Involved in OTM

In the early stages of OTM, the fluid in the periodontal ligament (PDL) cells is shifted, therefore causing strain on both the PDL and ECM. Applied mechanical forces are transduced from the strained extracellular matrix (ECM) to the cytoskeleton through cell surface proteins. This mechano-transduction occurs by ECM binding to cell adhesion molecules, known as integrins, and other cell surface receptors (Krishnan and Davidovitch 2005). The areas where the integrin receptors physically link actin-associated cytoskeletal proteins with the ECM and with adhesion molecules on adjacent cell surfaces are called focal adhesions. Integrin-mediated adhesive interactions play a key role in cell migration, proliferation and differentiation, but they also regulate intracellular signal transduction pathways. As such, integrins function as both cell adhesion molecules and intracellular signaling receptors (Meikle 2006). The strain produced by the mechanical forces stimulates a chain of events that plays a key role in the movement of teeth. First, there is a change in the shape of the cells in the ECM, and this elicits the release of signaling molecules from the affected cells. Due to the tension or compression of orthodontic forces, the nerve terminals of the ECM cells become distorted and release vasoactive neurotransmitters. Since the nerve terminals in the PDL cells are located near blood vessel walls, the neurotransmitters immediately recruit circulating leukocytes through diapedesis. Then, the leukocytes emit signaling molecules, including growth factors, colony-stimulating factors and cytokines, therefore causing an inflammatory response stimulating the remodeling of the PDL and alveolar bone cells (Krishnan and Davidovitch 2005). Substance P, an enkephalinic

neurotransmitter, also is released causing vasodilation, increased vascular permeability, and some analgesia. Greater forces will cause decreased release of substance P, therefore causing more discomfort to the patient (Parris *et al*. 1989).

Cytokines

Cytokines are low-weight extracellular signaling proteins produced by cells that control the action of other cells in a paracrine or autocrine fashion. They have various roles in the body, such as mediating the immunological response of the host to exogenous antigens, and both physiological and stress-induced bony metabolism. These proteins are produced by fibroblasts, osteoblasts, and other connective tissue cells (Meikle 2006). The secretion of these proteins is generated by various stimuli, including neurotransmitters, bacterial products, other cytokines, and mechanical forces (Krishnan and Davidovitch 2005). Cytokines are divided into groups according to their functions and include interleukins (IL), tumor necrosis factor (TNF), interferons (IFN), growth factors (GF), and colony-stimulating factors (CSF). Systemic hormones and mechanical stimuli, such as the forces generated in orthodontic tooth movement, influences bone metabolism by their ability to control the synthesis and action of cytokines (Meikle 2006). The cytokines that affect bone metabolism via orthodontic forces during OTM include interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor alpha (TNFα), gamma interferon (IFN$_\gamma$), and osteoclast differentiation factor (ODF) (Krishnan and Davidovitch 2005).

IL-1 is the most potent stimulator of osteoclast function, and it serves as a bone resorptive agent. The interleukins attract leukocytes, and stimulate target cells such as fibroblasts, endothelial cells, osteoclasts, and osteoblasts. The main role of the interleukins is to promote bone resorption and inhibit bone formation. Another pro-inflammatory cytokine, TNFα, induces both acute and chronic inflammation, and promotes bone resorption, while inhibiting bone formation. TNFα promotes osteoclastogenesis in tandem with its inflammatory response (Krishnan and Davidovitch 2009). IFN$_\gamma$, a strong inducer of major histocompatibility antigens in macrophages, promotes the synthesis of cytokines, such as IL-1 and TNFα. IFN$_\gamma$ also encourages bone resorption by apoptosis of effector-T cells (Krishnan and Davidovitch 2005; Meikle 2006).

Osteoclasts and Osteoblasts

Two major groups of cells control the body's bony metabolism. Osteoclasts and osteoblasts have equal but opposite functions during normal bone remodeling and skeletal homeostasis (Horowitz *et al*. 2001). However, in metabolic disease or extreme skeletal stresses and strains, the osteoblast to osteoclast ratio is altered, and the affected

area enters a state either of predominant bone resorption (*i.e.,* osteoporosis) or deposition (*i.e.,* osteopetrosis).

Osteoblasts are produced locally by the proliferation and differentiation of periodontal ligament fibroblast-like cells (Roberts *et al*. 1981). Therefore, osteoblasts are mesenchymal in origin, since they differentiate from mesenchymal stem cells through a series of progenitor stages, and finally form mature, matrix secreting osteoblasts (Horowitz *et al.* 2001). The main role of osteoblasts is to secrete bone extracellular matrix proteins that will become mineralized to form functional, strong, and mechanically sound bone. In addition to bone deposition, osteoblasts secrete matrix metalloproteinases (MMPs) to degrade the non-mineralized layer of osteoid prior to bone resorption. Without the removal of this organic layer, osteoclasts are unable to attach to the bone's surface to initiate bone resorption (Henneman *et al.* 2008).

Conversely, osteoclasts remove bone structure. These multi-nucleated giant cells are found only in bone and exclusively remove calcified structures (Suda *et al*. 1999). Specifically, osteoclasts are located on endosteal bone surfaces and the periosteal surfaces beneath the periosteum (Arai *et al.* 1999). Osteoclasts are hematopoietic in origin, and are strongly associated with macrophages (Horowitz *et al*. 2001). Bone resorption, whether it physiological or in response to external stimuli (*e.g.,* orthodontic force), is accomplished by the osteoclast binding to the bone surface and forming a zone sealed from the extracellular environment. Then, the osteoclast forms a resorption lacuna as a consequence of secreting proteolytic enzymes into the space between the bone and the osteoclast plasma membrane (Horowitz *et al*. 2001). The proteolytic enzymes remove the osteoid of the bone leaving the mineral content of bone exposed. Subsequently, the osteoclast uses a unique organelle, known as the ruffled border, to pump hydrogen ions (H^+) into the lacuna to dissolve the mineral component of the bone (Horowitz *et al*. 2001). In addition, the collagenous component of bone is destroyed by lysosomal cysteine proteinases and cathepsins, which are also secreted by the osteoclast (Sasaki 2003). Mature active osteoclasts are characterized by a high expression of markers, such as tartrate resistant alkaline phophatase (TRAP), cathespin K, calcitonin, and vitronectin receptors (Boyle *et al*. 2003; Feige 2001).

In the customary bone remodeling cycle, osteoclastic bone resorption proceeds osteoblastic bone formation. Therefore, osteoclasts are recruited to a specific site via cell signaling, adhere to the bone, and promote resorption. In opposition, osteoblasts identify the areas of resorption by an unidentified means, travel to the affected sites and repair the deficiency by secreting bone extracellular matrix (*i.e*., osteoid). The coupling action of these two cells maintains the equilibrium of bony skeleton (Horowitz *et al*. 2001). In regards to the directed, external forces used in OTM, osteoclastic bone resorption is essential, for the reason that the supporting alveolar bone must be removed from the pressure side of the tooth's PDL for tooth movement to occur (Masella and Meister 2006; Oshiro *et al*. 2003; Roberts *et al*. 1981). However, on the stretched side of

the PDL, osteoblastic bone formation must occur to keep the tooth well supported in the alveolar bone (Henneman *et al.* 2008; Masella and Meister 2006).

Osteoclast Differentiation and Activation

Osteoclastogenesis is a complex progression of events that requires intimate contact between the PDL cells (fibroblasts) and bone cells (osteocytes and osteoblasts) to form fully activated osteoclasts (Boyle *et al.* 2003; Henneman *et al.* 2008; Ikeda *et al.* 2000). The osteoclast is a tissue-specific macrophage polykaryon created by the differentiation of monocyte/macrophage precursor cells at or near the bone surface (Boyle *et al.* 2003). First in the presence of the cytokine M-CSF (macrophage – colony stimulating factor) the hematopoietic precursor cells, are stimulated to differentiate into pre-osteoclasts. Then, soluble factors produced by osteoblasts and fibroblasts aid in the further differentiation of pre-osteoclasts to mature osteoclasts (Boyle *et al.* 2003; Henneman *et al.* 2008). Mediators produced by fibroblasts and osteoblasts include M-CSF, receptor activator of nuclear kappa β ligand (RANKL), osteoprotegerin (OPG), and bone morphogenic proteins (BMPs) (Henneman *et al.* 2008). According to Arai *et al.* (1999), pre-osteoclasts can differentiate into osteoclasts in the presence of both M-CSF and RANKL, however in the presence of M-CSF only, they differentiate into macrophages. Cell-to-cell contact between stromal cells and osteoblasts expressing RANKL allow several pre-osteoclasts to fuse together to form a polykaryon, or multi-nucleated giant cell (Boyle *et al*. 2003). In the final step of osteoclastogenesis, the polykaryon attaches to the bone via specific integrins, and is stimulated by osteopontin (OPN) to complete differentiation (Henneman *et al.* 2008). Characteristically, osteoclasts show a high level of cell adhesion, and their growth and differentiation are anchorage dependent (Arai *et al.* 1999). As a result, an osteoclast does not become fully activated, or mature, until attached to bone. A mature osteoclast is capable of resorbing mineralized tissue, as well as producing enzymes to resorb the fibrous components of bone (Boyle *et al*. 2003; Henneman *et al.* 2008). The life span of an osteoclast is related to the duration of a positive stimulus. The osteoclast will remain active as long as positive stimulus is present (expression of RANKL or IL-1 by osteoblast/stromal cells). When down-regulation of RANKL or up-regulation of OPG occurs (*e.g.*, the stimulus is removed), osteoclasts will quickly experience apoptosis (Feige 2001; Suda *et al.* 1999). TNF-α presents another pathway of osteoclastogenesis, which is present in inflammatory bone resorption. Together with IL-1, TNF-α-induced osteoclasts are thought to play a role in the bone resorption seen in inflammatory bone resorption (Ikeda *et al.* 2001). Therefore, there are two pathways of osteoclastogenesis: the RANKL-pathway and the TNF-α pathway.

RANK, RANKL, and OPG

Both osteoblast and osteoclast differentiation and cellular activities are regulated by a variety of molecules, including (1) osteotropic hormones and cytokines, (2) inflammatory mediators, and (3) growth factors (Meikle 2006). The knowledge surrounding osteoclast differentiation and activation has been expanded upon by the modern analysis of the tumor necrosis factor (TNF) and TNF-like proteins, such as receptor activator of the nuclear factor – κβ (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) (Boyle *et al.* 2003; Leibbrandt and Penninger 2008).

The receptor activator of the nuclear factor kappa β ligand (RANKL), a TNF family member, is a membrane-bound protein that is expressed on osteoblasts, stromal cells, and other cell types (Feige 2001; Horowitz *et al.* 2001; Lacey *et al.* 1998). RANKL mRNA is expressed in many cell types throughout the body, including chondrocytes in developing bone and the periosteum of mature bone. The highest expressions of RANKL are seen in skeletal and primary and secondary lymphoid tissue (Leibbrandt and Penninger 2008). RANKL expression is increased by parathyroid hormone (PTH), $1\alpha 25\text{-}(OH)_2$ Vitamin D3, dexamethasone, IL-1, IL-11, oncostatin M, and PGE_2, although it is decreased by TGF-β (Horowitz *et al.* 2001).

Three isoforms of RANKL have been identified, named RANKL 1, RANKL 2, and RANKL 3. Each isoform is unique, yet all can mediate osteoclastogenesis. The originally discovered isoform, RANKL 1, is implicated in the roles of survival and activation of dendritic cells or T-cells and as a factor of osteoclastogenesis (Ikeda *et al.* 2001; Liebbrandt and Penninger 2008). Both RANKL 1 and RANKL 2 have transmembrane domains, but RANKL 2 has a shorter intracellular domain and is regulated by a different promoter than the other two isoforms. The soluble isoform, RANKL 3, does not have a transmembrane domain or intracellular domain and appears to be produced physiologically. The extracellular domains of all three isoforms are essentially identical and capable of binding to two different receptors, known as RANK and OPG, both of which are TNF-related receptor proteins. RANK was isolated from dendritic cells, while OPG was isolated as a protein inhibiting bone resorption (Ikeda *et al.* 2001). A study by Leibbrandt and Penninger (2008) stated that the membrane-bound RANKL isoforms induce osteoclastogenesis more efficiently than the soluble RANKL isoform.

RANKL, via intercellular signaling, has a potent effect on osteoclast differentiation from hematopoietic precursor cells, and it stimulates osteoclast bone resorptive activity (Burgess *et al.* 1999; Udagawa *et al*. 1999). Binding of RANKL to an osteoclast RANK site results in the activation of signaling pathways leading to the intracellular expression of several TNF receptor associated factors (TRAF's) (Boyle *et al.* 2003; Wise and King 2008). TRAF6 acts as a key adaptor to assemble signaling proteins for the NF-κβ pathway, which directs osteoclast-specific gene expression leading to

differentiation and activation (Boyle *et al.* 2003). Therefore, a TRAF6 deficiency will lead to osteopetrosis (Boyle *et al.* 2003; Burgess *et al.* 1999). The survival of the mature osteoclast depends on the presence of RANKL (IL-1 and M-CSF also promote osteoclast survival). Nevertheless, without it the osteoclasts quickly undergo apoptosis (Feige 2001; Suda *et al.* 1999). Another key regulator in osteoclastogenesis is M-CSF, a product of the stromal cells. In the presence of RANKL, M-CSF can stimulate the differentiation of osteoclasts from hematopoietic precursors and activate mature osteoclasts. Without RANKL, M-CSF cannot cause osteoclast differentiation, therefore causing the precursors to develop into macrophages (Arai *et al.* 1999; Burgess *et al.* 1999). M-CSF plays three roles in osteoclastogenesis: (A) it induces RANK; (B) it is a competence factor for differentiation; and (C) it stimulates cell survival and proliferation. As a result, RANKL is the differentiation factor for osteoclasts, but it is not an exclusive osteoclast commitment factor due to its expression in osteoclasts, dendritic cells, and T-cells (Arai *et al.* 1999).

On the other hand, OPG is a novel soluble member of the tumor necrosis factor receptor superfamily, which has profound inhibitory effects on osteoclast differentiation and bone resorption (Burgess *et al* 1999). The term osteoprotegerin literally means protector of bone, and it was the first molecule of the trio to be discovered by virtue of its ability to inhibit osteoclast development *in vitro* and *in vivo* (Liebbrandt and Penninger 2008). OPG, also known as osteoclast inhibitory factor, is found in the body only as a soluble *decoy* receptor, because it lacks a membrane-spanning domain (Feige 2001; Suda *et al.* 1999). In order to become active, the initial osteoprotegerin protein must cleave the amino acid signal peptide to become the mature functional peptide (Liebbrandt and Penninger 2009). Binding of OPG to the RANK site of preosteoclasts competitively inhibits the binding of RANKL, which terminates their differentiation into mature osteoclasts (Kanzaki *et al*. 2005). OPG is found in high concentrations in developing bone, and its expression is increased by bone morphogenetic protein (BMP), IL-1, TNF, TGFβ, and estrogen. Conversely, its production is inhibited by PGE_2, glucocorticoids, $1,25(OH)_2$ vitamin D3 and PTH (Horowitz *et al* 2001). Although OPG expression is typically found near areas of bone, it has also been found in vessel walls, and it may be responsible for maintaining large vessel viability. More importantly, OPG may regulate pericytes, which are responsible for the life-threatening osteoblastic-like calcification in the walls of blood vessels (Horowitz *et al.* 2001). OPG mRNA expression has also been found in the brain, liver, lung, heart, kidney, skeletal muscle, skin, intestines, calvaria, stomach, testis and placenta (Liebbrandt and Penninger 2008, Liebbrandt and Penninger 2009).

When added to bone marrow cultures, OPG reversibly inhibits osteoclastogenesis (Yasuda *et al.* 1998). For example, in mice treated with 10 mg/kg OPG intravenously all osteoclasts disappear within 48 hours (Feige 2001). However, within the next 7 to 10 days, osteoclasts return and can be found in normal numbers and in typical locations in these OPG-treated mice (Lacey *et al.* 1998). Given that treatment with

OPG reduces the number of osteoclasts and reversibly inhibits RANKL binding, it has been suggested that it could be used in the treatment of bone arthropathies, such as osteoporosis, crippling arthritis, and osteopenic disorders (Liebbrandt and Penninger 2008, Liebbrandt and Penninger 2009). A recent study determined that bone resorption modulated by RANKL and OPG not only stimulated osteoclast differentiation, but also affected changes in osteoblast proliferation, which is highly suggestive of a feedback mechanism from osteoclasts to osteoblasts (Lin *et al. 2007*).

RANK, or the receptor activator of NF-κB, is the functional receptor for both RANKL and OPG (Horowitz *et al.* 2001). RANK is a member of the TNF receptor superfamily, and it encodes for type I transmembrane glycoproteins that has four parts including a signal peptide, an extracellular domain, a transmembrane domain, and a cytoplasmic domain (Horowitz *et al.* 2001; Liebbrandt and Penninger 2008, Liebbrandt and Penninger 2009). Both RANK and OPG molecules competitively bind to RANKL, thereby inducing either bone resorption or bone deposition, respectively. Expression of RANKL and OPG is therefore coordinated to regulate bone resorption and density positively and negatively by controlling the activation state of RANK on osteoclasts (Boyle *et al*. 2003). Since RANK is a TNF receptor, it is assumed that RANK trimerization is a prerequisite for RANKL binding and signal transmission. RANK mRNA is most abundant in dendritic cells, bone, skeletal muscle, thymus, liver, colon, small intestine, and the adrenal gland, but RANK protein can also be detected on the surface of dendritic cells, $CD4^+$ and $CD8^+$ T-cells, Langerhans cells, or on mammary epithelial cells where expression is regulated throughout pregnancy (Horowitz *et al.* 2001; Liebbrandt and Penninger 2008, Liebbrandt and Penninger 2009) (**Figure 2**).

Osteoclast differentiation and function appear to be regulated by a counterbalancing system, which has been referred to as the RANKL/RANK/OPG regulatory axis (Boyle *et al.* 2003). The RANKL/OPG regulatory system is controlled by the regulation of specific gene expression, and serves the purpose of maintaining bone structure and function, as well as meeting the body's physiological needs for ions sequestered in bone (**Figure 3**). An increased RANKL/OPG ratio will favor osteoclast formation and activation, so bone resorption will occur. In contrast, a decreased RANKL/OPG ratio promotes bone formation by inhibiting osteoclastic activity (Kanzaki *et al.* 2001). Tight control over the body's RANKL/OPG regulatory axis must be maintained in order to keep bone remodeling in homeostasis (Boyle *et al.* 2003). For example, over-expression of OPG or treatment with recombinant OPG has shown an increase in bone density and osteopetrosis in mice. However, ablation of OPG expression showed early onset osteoporosis. On the other hand, mice with inactivated RANKL expression developed severe osteopetrosis and showed defective tooth eruption resulting from a complete lack of osteoclasts, while over-expression of RANKL led to osteoporosis (Feige 2001; Leibbrandt and Penninger 2008, Leibbrandt and Penninger 2009). These findings unambiguously establish the pivotal role of RANKL-RANK

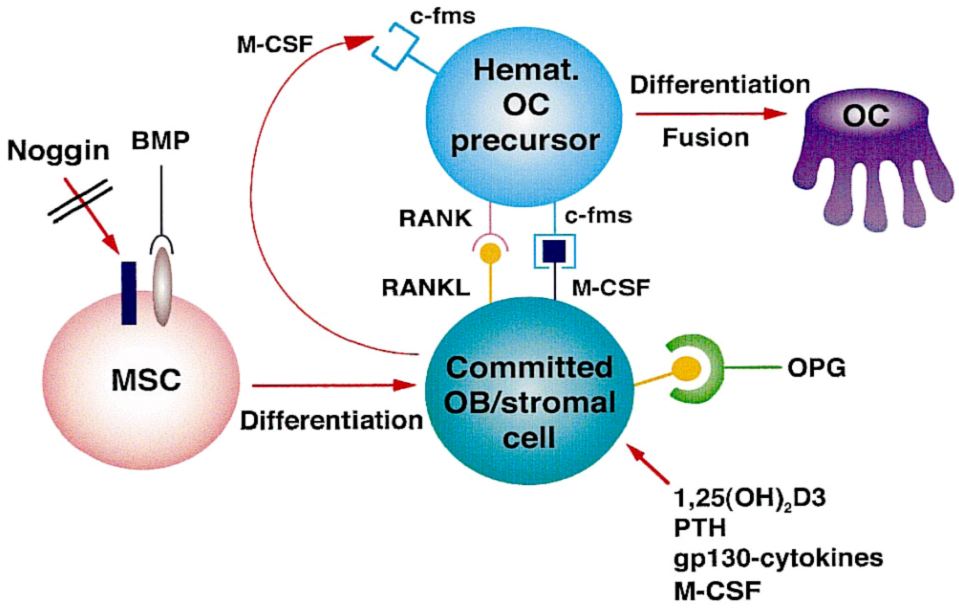

Figure 2. RANKL and OPG interactions on RANK in osteoclast development. The progenitors of stromal cells and osteoblasts are mesenchymal stem cells (MSC). After exposure to bone morphogenic proteins (BMPs), a MSC will differentiate into an osteoblast or a stromal cell. However, exposure to certain antagonistic proteins like Noggin impedes the differentiation process. Committed stromal cells and osteoblasts express RANKL on their cell surface. RANKL is regulated by a variety of molecules including 1,25(OH)$_2$D3, PTH, M-CSF, and gp130-receptor cytokines (such as IL-6 and Oncostatin M). RANKL interacts with its receptor, RANK, which is expressed on hematopoietic osteoclastic precursor cells. Interaction between RANKL and its receptor RANK induces terminal differentiation of these cells into mature bone resorbing osteoclasts, therefore promoting bone resorption. Conversely, OPG, also known as a decoy receptor, inhibits the RANKL-RANK interaction by binding to RANKL. OPG-RANKL binding terminates osteoclastogenesis and promotes bone deposition. M-CSF plays a vital role in osteoclast differentiation, but cannot fully activate mature osteoclasts without the presence of RANKL. Soluble M-CSF, secreted by stromal cells or osteoblasts, interacts with its receptor c-fms to support and enhance osteoclastogenesis. In addition, stromal cells and osteoblasts also express M-CSF on their cell surface. It has yet to be determined whether this form of M-CSF is active in osteoclast formation. (Horowitz *et al*. 2001).

Source: Reprinted with permission. Horowitz MC, Yougen X, Wilson K, Kacena MA. Control of osteoclastogenesis and bone resorption by members of the TNF family of receptors and ligands. Cytokine Growth Factor Rev 2001;12:9-18.

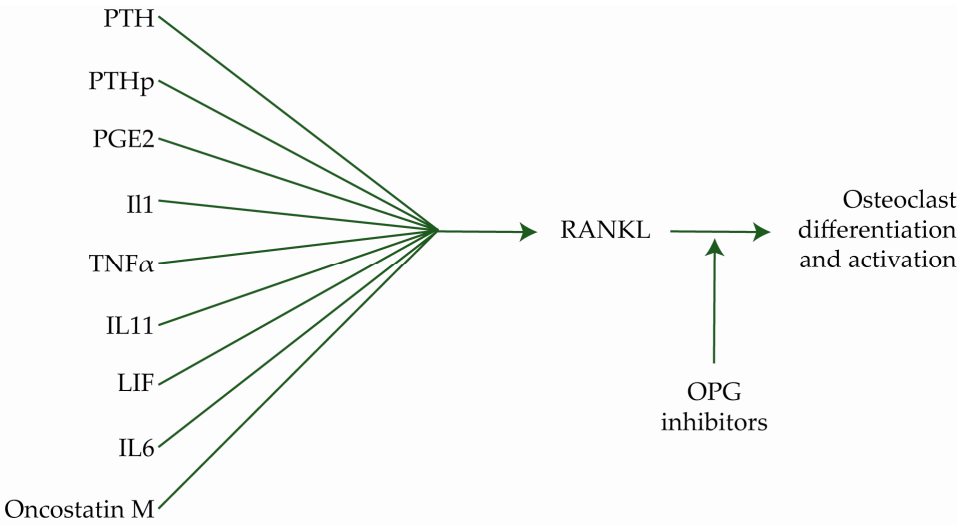

Figure 3. The balance between RANKL and OPG controls osteoclast activity. Feige (2001) and others suggest that most inducers of bone resorption—and of hypercalcemia—act through the RANKL-OPG axis**.**

Source: Reprinted with permission. Feige U. Osteoprotegerin. Ann Rheum Dis 2001;60:81-4.

interactions in positively regulating osteoclastogenesis, counteracted and balanced by OPG, which functions as a natural decoy receptor for RANKL (Boyle *et al.* 2003; Liebbrandt and Penninger 2008). In effect, all factors that either inhibit or enhance bone resorption by osteoclasts also positively or negatively influences RANKL and OPG mRNA/protein levels. Therefore, the complex process of osteoclast-mediated bone remodeling converges at the RANKL/RANK/OPG axis (Liebbrandt and Penninger 2008). The essential function of this regulatory axis in osteoclastogenesis, and bone remodeling also is relevant to human bone diseases. New, promising drugs targeting RANKL and OPG are being developed for the treatment of bone arthropies (Liebbrandt and Penninger 2009).

Functions and Dysfunctions of the RANKL/OPG Regulatory Axis

Tooth Eruption and Development

Several local signaling molecules have been discovered around the developing tooth bud, however RANKL, RANK, and OPG have been found at significant levels in these areas (Liu *et al.* 2005). At times of increased osteoclastic activity, the RANKL/OPG ratio is increased in one of two ways: (1) down-regulation of OPG by CSF-1; or (2) by up-regulation of RANKL by a number of osteogenic cytokines such as TNF-α, IL-1α, TGF-β1, and CSF-1. Studies of RANKL knockout mice have shown that absence of RANKL leads to failed tooth eruption, even when given a RANKL transgene, suggesting that the RANKL needed to initiate and sustain alveolar bone resorption is produced by the dental follicle (Liu *et al*. 2005). RANKL is expressed primarily in the coronal part of the dental follicle, while OPG is thought to predominate in the apical portion, favoring bone formation (Harokopakis-Hajishengallis 2007).

Ohazama *et al*. (2004) also examined OPG, RANK, and RANKL gene expression during tooth development in mice. Gene expression was analyzed in explant cultures from epithelial thickening to cytodifferentiation stages. OPG is weakly expressed in the initial thickening of tooth epithelium, as well as in the outer edges of the mesenchyme during the bud stage. RANKL and OPG are both strongly expressed in the internal and external enamel epithelium throughout the cap stage. When RANKL signaling is temporarily inhibited during tooth development by administration of exogenous OPG, adverse effects are observed. Tooth development is delayed, resulting in thin dentin and enamel and narrower pulp tissue. This suggests that the RANKL signaling system plays an integral role in tooth development, and disruption or dysfunction of the RANKL/OPG axis can have serious negative consequences.

Primary Tooth Root Resorption

In order for tooth eruption or orthodontic tooth movement to occur, two requirements must be met. First, both of these processes require a soft tissue, such as the PDL, that intervenes between the tooth structure and the alveolar bone. This soft tissue plays an important role in regulating the remodeling of the adjacent tissues. Second, both require bone turnover that is temporarily and spatially regulated to facilitate specific translocations of teeth through the alveolar bone (Wise and King 2008).

Tooth eruption is a fundamental developmental and physiological process, while force plays a secondary role. Resorption of the primary tooth root is a requisite for the proper eruption and emergence profile of its permanent successor (Wise and King 2008). The bilateral consistency and symmetry regarding the exfoliation of primary teeth and the eruption of permanent teeth suggest that these occurrences are coupled and genetically programmed events. The pressure of the erupting tooth has been reported as the initial contributory action in the chain of events leading to primary tooth exfoliation (Sahara 2001). However, a permanent successor is not mandatory, because the roots of primary teeth without a permanent successor will eventually resorb as well. The eruption and exfoliation process is regulated by the endocrine system and the nutritional intake of the individual (Harokopakis-Hajishengallis 2007); therefore these factors have an indirect effect on the resorption course of the primary root. Conditions such as hypothyroidism, pituitary dwarfism, and chronic malnutrition will usually delay the shedding of primary teeth, and these conditions can interfere with the eruption of permanent teeth (Harokopakis-Hajishengallis 2007).

Root resorption of the primary teeth starts at the site of the root closest to its permanent successor. Root resorption is initiated and regulated by the stellate reticulum and the dental follicle of the underlying permanent tooth via the secretion of stimulatory molecules (Harokopakis-Hajishengallis 2007). Molecularly, the root resorption process is similar to bone remodeling, involving many of the same transcription factors and cytokines. Release of RANKL from a tooth's dental sac seems integral to the dissolution of bone occlusal to the permanent tooth and, thus, bone resorption is a rate-limiting factor in tooth eruption (at least during the preemergent phase) (Tyrovola *et al.* 2008). Likewise, RANKL from the succedaneous tooth's dental sac activates odontoclasts, leading to root resorption and the subsequent exfoliation of the primary predecessor (Harokopakis-Hajishengallis 2007; Tyrovola *et al.* 2008). Odontoclasts are responsible for the resorption of dental hard tissue. Compared to osteoclasts, odontoclasts are smaller in size, have fewer nuclei, and produce smaller resorption lacunae. The metabolic and enzymatic properties of odontoclasts are also similar to osteoclasts (Harokopakis-Hajishengallis 2007; Tyrovola *et al*. 2008; Yildirim *et al.* 2008). RANKL and CSF-1 have been found in much higher concentrations than OPG during *in vivo* studies of the dental pulp of exfoliated primary teeth, which also might contribute to primary tooth root resorption and exfoliation (Yildirim *et al.* 2008).

External Apical Root Resorption (EARR)

External apical root resorption is an unavoidable pathologic consequence of orthodontic tooth movement. EARR is defined as an iatrogenic disorder that occurs, unpredictably and most commonly during orthodontic treatment, whereby the resorbed apical root portion is replaced with normal bone (Yamaguchi *et al.* 2006). EARR is a complex, sterile inflammatory process; however certain risk factors increase an individual's likelihood of developing this condition. Individual susceptibility, genetics, and systemic factors are risk factors for developing EARR (Harris *et al.* 1997; Yamaguchi *et al.* 2006), while sex and age during treatment do not predispose a person to an increased risk of EARR (Harris 2000; Hartsfield, Everett and Al-Qawasmi 2004). Other predictors for EARR include: short blunted roots, irregular root form, trauma, tooth devitalization with or without endodontic therapy, ectopic eruption, traumatic occlusion, heavy mastication and bruxism, and oral habits such as tongue thrusting and nail-biting (Harris 2000; Hartsfield, Everett and Al-Qawasmi 2004; Parker and Harris 1998). The movement of teeth with mature roots, extensive root movement, and intrusive mechanics have been reported to increase the risk of EARR (Parker and Harris 1998).

Variations in RANKL and OPG expression play an important role in root resorption (Boyle *et al.* 2003). Odontoclasts and cementoblasts share similar regulatory pathways with osteoblasts and osteoclasts (Oka *et al*. 2007; Sasaki 2003), so there is reason to suppose that increased expression of RANKL is associated with severe root resorption (Al-Qawasmi *et al*. 2006). In a rat study model by Low *et al.* (2005), increased expression of RANKL was seen in the presence of root resorption induced by heavy orthodontic forces. Compressed PDL cells extracted from patients exhibiting severe root resorption expressed significantly more RANKL and significantly less OPG compared to patients exhibiting normal root resorption (Yamaguchi *et al.* 2006). This suggests that the compressed PDL cells in cases of severe EARR may produce large amounts of RANKL, and therefore up-regulate osteoclastogenesis (Tyrovola *et al.* 2008). It has been suggested that up to 90% of individual variation in observed root resorption can be attributed to types of mechanics (*e.g*., intrusion, lingual root torque) (Harris *et al.* 1997), but individual variation in RANKL/OPG expression may also result in variations in observed clinical root resorption. Moreover, individual responses to specific vectors (*e.g*., intrusion, lingual root torque) are themselves modulated by the person's genotype (Hartsfield *et al.* 2004).

Bone Metabolism Dysfunction

The RANKL/OPG system is responsible for regulating a wide variety of biological and physiological processes, many with major health-care ramifications. Dysfunction resulting from alterations in the RANKL/OPG ratio accounts for a number

of abnormalities in bone dynamics. For instance, if the ratio of RANKL to OPG is increased (by either an over-expression of RANKL or a deficiency of OPG), osteoclastic resorption of bone will predominate over osteoid deposition by osteoblasts, and osteoporosis will develop (Sasaki 2003). Pharmacological companies and researchers alike are trying to develop new treatments and medications aimed at inhibiting RANKL mediated activation of osteoclasts by OPG. In theory, these new treatment regimens would be an effective method of treating the symptoms of osteoporosis, and may also lessen the amount of cartilage destruction seen in arthritis (Anandarajah 2009; Jones *et al*. 2002; Liebbrandt and Penninger 2007; Vega *et al.* 2007). In contrast, mice with a RANKL deficiency or OPG over-expression exhibit osteopetrosis, defects in tooth eruption, and few osteoclasts due to the inability of osteoblasts to initiate osteoclastogenesis through RANKL/RANK signaling (Kong *et al*. 1999). In the disease process of multiple myeloma, RANKL is up-regulated in the bone marrow microenvironment. It has also been observed that myeloma cells express RANKL and inhibit OPG expression, leading to osteoporosis, lytic bone lesions, hypercalcemia, and increased risk of bone fractures accompanied by severe bone pain (Leenheer 2004). Alterations in the RANKL/OPG ratio are also observed in the pathological process of Paget's disease (Rifkin and Gay 1992). Interestingly, the RANKL-OPG axis has been implicated in the slowed wound healing seen in type II diabetics, chronic heart disease (including calcifications of the blood vessels), and rheumatoid arthritis (Jones *et al*. 2002; Vega *et al.* 2007;). Recently, serum OPG levels have been used as a potential indicator in the diagnosis of certain disease processes; such as prostate cancer (marked increase in serum OPG levels), multiple myeloma (lower than normal concentrations of serum OPG), breast cancer (increased serum OPG levels may increase the tumor cell survival by inhibiting TRAIL-induced apoptosis), and renal disease (decreased serum OPG levels) (Vega *et al.* 2007). Therefore, it is clear that the RANKL/OPG system is responsible for regulating many biological processes and that preventing or correcting alterations in the system could help alleviate the symptoms of a number of pathological conditions.

Periodontitis in Smokers

Periodontitis is a chronic inflammatory disease that is caused by an infection of anaerobic, gram-negative bacteria (Buduneli *et al.* 2008; Lappin *et al.* 2007). The bacteria colonize in the subgingival area, and they cause localized and systemic elevations of pro-inflammatory cytokines (Buduneli *et al.* 2008). The increase in inflammatory mediators results in tissue destruction leading to gingival inflammation, attachment loss, bone loss, and eventually tooth loss if left untreated (Tang *et al.* 2009). The function of osteoclasts, which cause resorption of alveolar bone in periodontal disease, is regulated by interaction with periodontal ligament cells. The periodontal ligament fibroblasts appear to be involved in both stimulatory and inhibitory processes. However, gingival fibroblasts have been shown to produce higher levels of OPG and may have a greater protective effect than the periodontal ligament fibroblasts (Lappin *et*

al. 2007). Overall, bone resorption will occur in periodontitis as the result of the uncoupled process in bone remodeling, and it is usually reflected in an increased RANKL/OPG ratio. The increased ratio is either due to an increase in RANKL, a decrease in OPG, or a combination of both (Tang *et al.* 2009). Patients with diagnosed periodontitis have been reported to have an increase in RANKL and a decrease in OPG in both the gingival tissues and in gingival crevicular fluid. As a consequence, RANKL/OPG ratios are found to be increased in periodontitis patients when compared to healthy controls (Buduneli *et al.* 2008; Lappin *et al.* 2007; Tang *et al.* 2009).

One of the major risk factors for chronic periodontitis is smoking, which modifies the periodontal response to microbial challenge (Buduneli *et al.* 2008). A clear dose-response relationship between periodontitis and smoking has been reported in several studies (Tang *et al.* 2009). The relative risk of developing periodontitis increases by up to six times in smokers (Lappin *et al.* 2007; Tang *et al.* 2009). The periodontal pathogens for both smokers and non-smokers are similar; so the accentuated loss of alveolar bony support in smokers must be caused by nicotine actions on the body (Buduneli *et al.* 2008). Recently, several studies have investigated the link between RANKL and OPG and increased bone loss in smokers versus non-smokers with periodontal disease. Cigarette smokers tend to have lower serum concentration of OPG, which affects the RANKL/OPG ratio in a manner that lends itself to bony resorption. The increased bone loss seen in smoker-related periodontitis may be partially explained by suppression of OPG production (Buduneli *et al.* 2008; Lappin *et al.* 2007; Tang *et al.* 2009).

Gingival Crevicular Fluid

Gingival crevicular fluid (GCF) arises from the gingival plexus of blood vessels in the gingival corium, subjacent to the epithelium lining the dentogingival space (Alfano 1974). Components found within the GCF originate from the blood, host tissues, and subgingival plaque (Delima and Van Dyke 2003). The molecules isolated from the sulcular fluid include: electrolytes, small organic molecules, proteins, cytokines, specific antibodies, bacterial antigens, and enzymes of both host and bacterial origin (Carranza and Newman 1996; Delima and Van Dyke 2003). Gingival crevicular fluid contains several cell population such as; bacteria from the adjacent plaque mass, desquamated epithelial cells, and transmigrating leukocytes, and sometimes erythrocytes. However, red blood cells are an incidental finding, and they are indicative of injury to the microvasculature and inflammation (Delima and Van Dyke 2003).

When first discovered, crevicular fluid was considered a continuous transudate (Brill 1969), but it is currently considered an inflammatory exudate (Carranza and Newman 1996). In healthy gingival tissue, negligible amount s of GCF can be collected (Carranza and Newman 1996; Delima and Van Dyke 2003). Inflammatory conditions, such as gingivitis and periodontal disease, will increase the presence of the

inflammatory exudate by more than 5-fold (Kowashi *et al.* 1980). Nearly half of the cells in the sulcular fluid are leukocytes, thus the major route of entry into the oral cavity for these cells is by way of the gingival sulcus. These inflammatory cells persistently emigrate from the peripheral blood stream into the gingival sulcus due to chemotactic factors initiated by inflammation (Delima and Van Dyke 2003).

Orthodontic forces produce an environment that can be described as "a continuous sequence of inflammation and repair designed to restore normal tissue continuity and function" (Meikle 2006). As opposed to the inflammation observed in response to periodontal disease, this *inflammation* is aseptic, but the reaction involves many of the same cytokines because orthodontic tooth movement results from a combination of both pathologic and physiologic responses (Wise and King 2008). Iwasaki *et al*. (2005) measured changes in IL-1β and IL-1 receptor antagonist (IL-1RA) expression in human crevicular fluid in response to orthodontic tooth movement.

Obtaining RANKL and OPG from Crevicular Fluid

Gingival crevicular fluid can be collected from the gingival sulcus with minimal patient discomfort; however, the obstacle in obtaining the fluid is the relative shortage of it in the sulcus (Carranza and Newman 1996). The most common collection method includes the use of absorbent paper strips. Paper strips are placed into the sulcus until resistance is encountered. Proper placement of the paper strip is essential, because excess force during placement can cause irritation of the epithelium, which can trigger the discharge of a surplus of crevicular fluid (Carranza and Newman 1996). According to Löe and Holm-Pederson (1965), in order minimize the induced irritation; the paper strip should be placed immediately adjacent to the entrance of the sulcus.

As discussed in research in periodontology, RANKL and OPG are both present in gingival crevicular fluid, and they can be readily obtained clinically (Bostanci *et al*. 2007; Mogi *et al*. 2004). Consequently, these studies indicated that GCF samples can be collected noninvasively and longitudinally across time in the same subjects. Studies show that GCF containing measurable amounts RANKL and OPG can be obtained from the sulcus of teeth subjected to orthodontic forces (Heinrich *et al*. 2005; Kawasaki *et al*. 2006; Nishijima *et al*. 2006).

CHAPTER 3. MATERIALS AND METHODS

Patient Selection

In regards to RANKL and OPG levels in the gingival crevicular fluid (GCF), little knowledge seems to be available describing the variations according to age, race, and sex. In this study, 54 subjects were used to explore the possible differences between sex and age (**Table 1**). However a number of the participants were still undergoing growth and development, while another sample of the participants were only experiencing the static changes of bony remodeling associated with adulthood. The literature suggests that the decrease in the amount of tooth movement with age may be associated with a decrease in the RANKL/OPG ratio during the early stages of orthodontic tooth movement. The transition into adulthood induces a shift in the expression of RANKL and OPG that favors osteoclast formation, and it has been reported in humans that OPG levels decrease significantly with ageing (Tyrovola *et al.* 2008). An effort was made to achieve a proportionate number of males and females in both groups in this study. As is common, the recruitment of females in this study was greater than that of males. To evaluate the potential difference in RANKL and OPG production between races, subjects were identified either as white of European extraction or African American. An effort was made to obtain equal numbers of American whites and African American participants, but, due to the demographics of the patient pool in the school and the geographical location of the study, there were a slightly greater number of African Americans than American whites.

To minimize the variability of RANKL and OPG in the gingival crevicular fluid (GCF), there were several exclusion criteria. Subjects did not (1) have a remarkable medical history for a systemic disease; (2) did not have active periodontal disease or previous treatment for periodontal disease; (3) did not have acute gingival inflammation; (4) did not have untreated decay; (5) did not have any endodontic lesions; (6) did not have trauma from occlusion; (7) were not currently using any form of NSAIDS; and (8) were not currently using bisphosphonates. A recent study by Menezes

Table 1. Sample sizes by age, and race among the 54 participants in the study.

Age	n	Male	Female	Caucasian	African American
Child	26	18	8	11	15
Adult	28	8	20	13	15
Total	**54**	**26**	**28**	**24**	**30**

et al. (2008) showed that RANKL/OPG levels are affected by the development of periapical granulomas, which is the reasoning behind omitting subjects with radiographic pulpal disease. According to the Menezes study, radiolucencies smaller than 5 mm had a higher RANKL/OPG ratio than those with larger bony defects, which was indicative of the stable or progressive nature of the lesion. Previous studies on the cytokine levels in the GCF have described an increased ratio of RANKL to OPG during periodontal disease leading to bone loss (Bostanci *et al.* 2007), so subjects with active or previous periodontal disease were omitted from participation. NSAIDS are anti-inflammatory pharmacological substances that inhibit prostaglandin production. These inhibited prostaglandins are responsible for up-regulation of osteoclastic activities. Since NSAIDS eliminate the production of critical inflammatory cytokines, their use in orthodontics causes a noticeable reduction in clinical tooth movement (Arias and Marquez-Orozco 2006). Therefore, since the use of NSAIDS affects the RANKL to OPG ratio, potential subjects using these agents were not allowed to participate in the study. Acetaminophen is notably the only common analgesic that has been shown not to adversely affect the inflammatory process leading to clinical tooth movement (Roch *et al.* 1997). The positive pain relieving effect achieved by acetaminophen coupled with the lack of interference of prostaglandin synthesis may make this drug ideal for the discomfort experienced during orthodontic treatment.

Similarly, clodronate, a non-n-containing bisphosphonate has been shown to reduce tooth movement in rats by inhibiting the stress-induced gene expression of COX-2, which reduced the production of prostaglandin E_2 and RANKL (Liu *et al*. 2006) Although not all of the bisphosphonate class of drugs has been studied in regards to the RANKL and OPG ratio, individuals using any bisphosphonate drug were not allowed to participate in the study due to potential interaction with the specific cytokines being examined.

No substantial data can be found to determine the needed sample size to distinguish a difference in sexual dimorphism. Therefore, a subjective sample size of 54 subjects was used with 26 of the subjects being male and the remaining 28 being female. This arbitrary number surpasses the number of rats or humans used in previous experimental studies, and it is within the confines of the cost, time, and effort that can be expended during this study.

The study was HIPPA compliant, and IRB approval was provided before the study began (IRB # 08-08807-XP). The subjects were selected through an open enrollment process at the University of Tennessee, Department of Orthodontics. The majority of participants was either employees of the dental school or was selected from various screening appointments for comprehensive orthodontics. The subjects obtained from screening appointments were not required to begin comprehensive treatment during the research period. The accompanying parent or caregiver of the screened adolescent was also offered the opportunity to be a participant in this study, given that

the adult patient pool at the University of Tennessee is small. Many of the parents and siblings of the screened individuals accepted the offer to join the study. All subjects were recruited between June and November of 2010.

Orthodontic Force Model

After patient recruitment and obtaining informed consent, each patient had a transpalatal spring with constant force placed across their maxillary arch. The spring was ligated to buccally-bonded fixed retainers on the maxillary first and second premolars (**Figure 4**). The orthodontic force model was placed with the following protocol:

1) The buccal surfaces of both maxillary first and second premolars were pumiced to remove any debris that could interfere with the bonding process.

2) The premolars were rinsed and air-dried. The teeth were isolated from saliva contamination by cheek retractors and cotton rolls.

3) The buccal surfaces of the four maxillary premolars were etched with 37% phosphoric acid gel for 60 seconds.

4) Each tooth was rinsed for a minimum of 10 seconds to ensure the complete removal of all the etchant.

5) The premolars were again air-dried, and fresh cotton rolls were placed to keep the teeth isolated from saliva contamination.

Figure 4. Occlusal and right and left lateral views of the nickel titanium transpalatal spring.
A transpalatal spring was attached bilaterally to bonded retainers on the maxillary premolars to allow a force of 150-250 grams to be exerted on the teeth.

6) The buccal surface of each premolar was then coated with a thin layer of TransBond XT® bonding primer. The primer was light-cured for 5 seconds on each tooth.

7) A sufficient amount of TransBond XT® adhesive paste was applied to the buccal surface of each tooth.

8) The fixed retainer was placed on top of the adhesive paste, and carefully situated in place below the height of contour of each tooth.

9) Excess adhesive paste was removed, and a small amount of bonding primer was rubbed over the surface of the fixed retainer and adhesive paste interface to ensure a smooth surface.

10) The adhesive paste on each tooth was light-cured for a minimum of 20 seconds.

11) Following the bonding procedures, the cheek retractors and cotton rolls were removed and the transpalatal spring was placed.

12) The transpalatal spring was held in place by 0.010" steel ligatures running under the contacts and through the embrasures of the adjacent premolars. The ligatures were securely ligated to the fixed retainers on the buccal surfaces of the premolars. A spring gauge was used to measure the initial loading of the spring, and the force placed upon the teeth was checked at each time interval while the spring was in place.

13) Each patient was given orthodontic wax to help minimize the discomfort of the appliances and to increase the likelihood of completion of the experiment.

14) Following the fourth sample collection, the appliance was completely removed. Any remaining adhesive was removed, and the buccal surfaces of the premolars were polished with a standard prophy paste.

Gingival Crevicular Fluid Sampling Protocol

Gingival crevicular fluid was sampled from both the pressure and the tension surfaces. Collection of samples separately from these two surfaces of the stressed premolars allows testing of the RANKL and OPG levels to determine if the concentrations are reversed during the opposing conditions (Garlet *et al*. 2008). Samples were stored at -20° C for later processing. The samples were collected at the following time intervals (**Figure 5**):

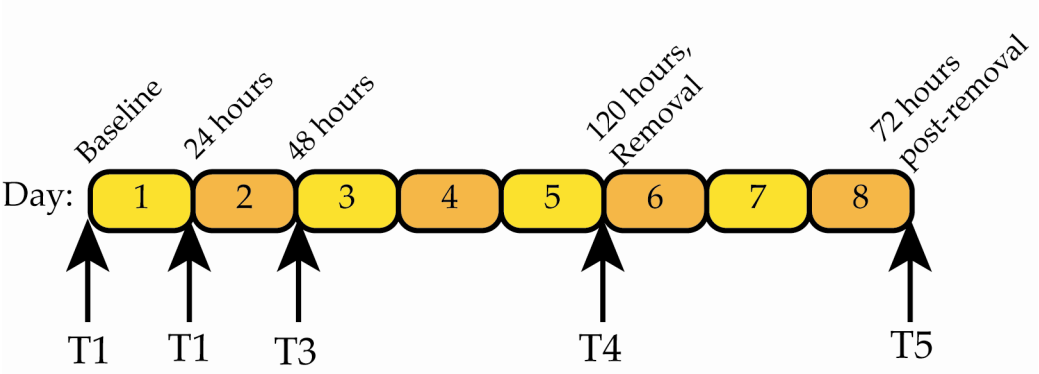

Figure 5. Schematic showing of the time-line of the experiment.
Each box is 1 day, with a total of 8 days.

1) A baseline sample was collected prior to the placement of any orthodontic appliance (T1). A transpalatal spring (TPS) was placed after sampling. Following the protocol of Parris *et al*. (1989), a spring gauge was used to measure the initial tension load of the TPS (in the range of 150 to 250 gm).

2) One day (24 hours) after TPS placement (T2). A spring gauge was used to re-set the tension of the TPS to that measured for the patient at T1 as needed.

3) Two days (48 hours) after TPS placement (T3). Tension of the TPS was re-set to that patient's T1 value.

4) Five days (120 hours) after TPS placement (T4). Spring tension was measured, and then the spring was removed after collecting the sample.

5) Three days (72 hours) after TPS removal, which was 8 days after the baseline sample (T5). No orthodontic treatment began until after the experimental period was concluded; this included spacers, bands, and brackets.

Sampling and Processing Preparation

1) At each reading, all clinically detectable supragingival plaque was removed from the maxillary first premolars without touching the gingival tissue to prevent plaque contamination. It was necessary not to touch the gingiva to prevent blood contamination.

2) Saliva contamination was prevented by cotton roll isolation. The tooth also was also air-dried to aid in prevention of saliva contamination.

3) A paper collection strip (PerioPaper strips – *Oraflow* Inc., Smithtown, NY, product # 593520) was inserted 1 mm into the gingival sulcus on the pressure and separately on the tension sides (**Figure 6**).

4) If any strips were noted to be contaminated with blood upon removal, they were discarded.

5) The Periotron® (Ora*flow* Inc.) was used to measure the amount of gingival crevicular fluid collected from each site. The Periotron reading from each paper strip was converted to μL via of the formulations published by Chapple *et al*. (1995).

6) The paper strips were combined together into (4 from the pressure sides and 4 from the tension sides) one 1.5 mL centrifuge tube containing 200 μL phosphate buffered saline (PBS, pH 7.2) and protease inhibitors: 0.1 mM phenylmethylsulphonylfluoride and 5 μg/mL each of leupeptin, pepstatin, amastatin, chemostatin, and antipain (made from Protease Single-Use Cocktail by Thermo Fisher Scientific, Inc., Waltham, MA). The sample extraction was allowed to take place for 2 hours at 4° C prior to re-freezing the samples.

7) The samples were stored at -20° C for later processing.

8) The samples were then thawed and gently shaken for 1 minute. The tubes were then centrifuged at 2000X g for 1 minute at 4° C.

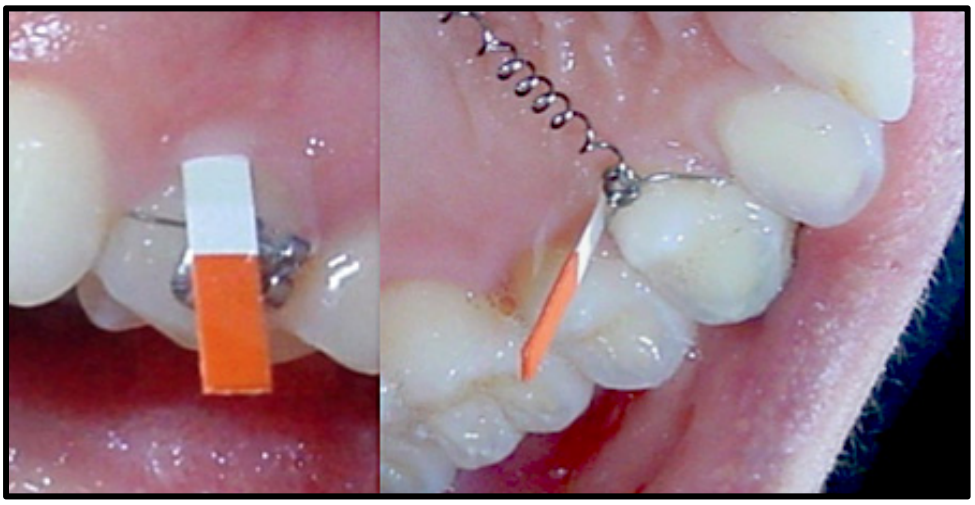

Figure 6. Insertion of the perio paper 1 mm into the sulcus.
The perio paper was inserted into the sulcus and remained in place for 60 seconds to absorb the highest possible volumes of GCF.

9) The volume of sample collected was recorded and additional buffer was added to each sample to dilute the total sample volume to 200μL. Since different amounts of buffer were added to each sample, the dilution factor was calculated for every sample to allow accurate calculation of RANKL and OPG concentrations.

10) The samples were then processed with enzyme-linked immunosorbent assays (ELISA) kits for RANKL (PeproTech, Rocky Hill, NJ # 900-K142) and OPG (R & D Systems Inc., Minneapolis, MN # DY805) analysis.

RANKL ELISA Processing

1) 100 μL of capture antibody were added to each ELISA plate well. The plate was sealed and allowed to incubate overnight at room temperature.

2) The plate was washed four times with wash buffer and inverted on a paper towel to allow the residual wash buffer to drain our of the individuals wells. Excess wash buffer was removed by blotting the plate against dry paper towels.

3) 300 μL of block buffer were added to each ELISA plate well. The block buffer was allowed to incubate for a minimum of an hour at room temperature.

4) Another wash cycle was completed following the block buffer incubation (see above #2).

5) Standards were prepared according to normal dilution. 50 μL of both the standards and samples were added to each well in duplicate. Triplicates were not used during this study due to the small amount of GCF collected. Minimal amounts of the extraction buffer were added to each sample in order to decrease the likelihood of over-dilution; and therefore, a negligible ELISA reading.

6) After a 2 hour incubation period for the standards and samples, another wash cycle was completed (see above #2).

7) 50 μL of detection antibody were added to each ELISA plate well. The detection antibody was allowed to incubate for two hours.

8) Another wash cycle was completed following the detection antibody (see above #2).

9) 50 μL of avidin peroxidase was added to each ELISA plate well. The avidin peroxidase was allowed to incubate for 30 minutes. During this incubation period, the ELISA plate was placed in drawer to prevent the sunlight from interfering with the occurring reaction.

10) Another wash cycle was completed following the incubation of the avidin peroxidase (see above #2).

11) The ABTS substrate liquid was allowed to warm to room temperature prior to use. Then, 50 μL of avidin peroxidase was added to each ELISA plate well. The plate was allowed to incubate in a drawer, and it was monitored for color development of color.

12) The plate was read by an ELISA plate reader set a wavelength of 405 nm. The plate was read at 10 minutes, 20 minutes, and 30 minutes to determine the best fit of the standard curve.

13) Both the 4-parameter and the log/log-X curves were analyzed to determine the best fit for the assay. The 4-parameter graph was determined to have the best standard curve for the RANKL assay.

OPG ELISA Processing

1) 100 μL of capture antibody were added to each ELISA plate well. The plate was sealed and allowed to incubate overnight at room temperature.

2) The plate was washed three times with wash buffer and inverted on a paper towel to allow the residual wash buffer to drain out of the individuals wells. Excess wash buffer was removed by blotting the plate against dry paper towels.

3) 300 μL of reagent diluent were added to each ELISA plate well. The reagent diluent was allowed to incubate for a minimum of an hour at room temperature.

4) Another wash cycle was completed following the reagent diluent incubation (see above #2).

5) Standards were prepared according to normal dilution. 50 μL of both the standards and samples were added to each well in duplicate. Triplicates were not used during this study due to the small amount of GCF collected. Minimal amounts of the extraction buffer were added to each sample in order to decrease the likelihood of over-dilution; and therefore, a negligible ELISA reading.

6) After a 2 hour incubation period for the standards and samples, another wash cycle was completed (see above #2).

7) 50 μL of detection antibody were added to each ELISA plate well. The detection antibody was allowed to incubate for two hours.

8) Another wash cycle was completed following the detection antibody (see above #2).

9) 50 μL of a working dilution of Streptavidin-HRP was added to each ELISA plate well. The Streptavidin-HRP was allowed to incubate for 20 minutes. During this incubation period, the ELISA plate was placed in drawer to prevent the sunlight from interfering with the occurring reaction.

10) Another wash cycle was completed following the incubation of the Streptavidin-HRP (see above #2).

11) 50 μL of substrate solution were added to each ELISA plate well. The substrate solution was allowed to incubate for 20 minutes. During this incubation period, the ELISA plate was placed in a drawer to prevent the sunlight from interfering with the color development.

12) 25 μL of stop solution were added to each ELISA plate well. The plate was gently tapped following the addition of the stop solution to ensure thorough mixing.

13) The plate was immediately read by an ELISA plate reader set a wavelength of 450 nm.

14) Both the 4-parameter and the log/log-X curves were analyzed to determine the best fit for the assay. The log/log-X graph was determined to have the best standard curve for the OPG assay.

Five subjects voluntarily withdrew from study due to patient compliance issues. These participants either could not physically or emotionally keep the orthodontic force model in place for the required time period. The initial samples collected from these subjects were used to determine the appropriate dilution factors and standard analysis procedures for the RANKL and OPG assays.

Statistical Analysis

During this protocol, the same individuals were followed over a period of time (*i.e.*, a longitudinal study). A longitudinal study is the strongest of all research protocols; as a result a repeated-measures design can be used that is efficient for detecting differences if they truly exist. The correct variance-covariance matrix calculations are available using the SAS statistical packaged. Tests were evaluated as two-tail t-tests at the conventional alpha level of 0.05.

Statistically speaking, the primary question was whether the level of RANKL and/or OPG changes within individuals of different age groups over time given the

protocol of (1) inducing mechanical stress on the PDL and adjacent bone and then (2) removing the mechanical stress and testing for a recovery of the RANKL/OPG ratio to baseline levels. The basic statistical model was the analysis of covariance, or ANCOVA. Prior studies suggest that there is considerable inter-individual variability and that the distributions may not be normal because of outliers (*e.g.*, Hamman *et al.* 2010; Stutzmann and Petrovic 1984; Stutzmann and Petrovic 1989). Our two-fold expected solution was (1) to log-normalize the data (*e.g.*, Sokal and Rohlf 1995) so the effect of outliers is diminished and (2), in complementary analyses, use simpler nonparametric statistics to test for differences across time (*e.g.*, Siegel and Castellan 1998). Patient's age, sex, and race were evaluated as covariates, but we did not expect sex to contribute significantly to the observed variance. It is wholly unknown whether force of the TPS (in the range of 150 to 250 gm) would provide a discernible dose-response effect; we are unaware of any study that has tested for this. Inclusion of a range of tensional forces will allow us to test for a relationship using the ANCOVA model (with force and force^2 as covariates). Alternatively, we also used age, sex, force and force-duration as potential predictor variables in a stepwise logistic model (Freund and Littell 1991), but interpretation was the same as with the ANCOVA model.

We analyzed two outcome variables, namely the level of RANKL and that of OPG, because the titers of these two molecules are not tied together in any deterministic fashion. On the other hand, it seemed conventional in the literature to rely on the ratio of RANK-to-OPG, so changes in this proportionality also will be evaluated.

CHAPTER 4. RESULTS

Tooth Movement Model

The research protocol used here is detailed in the prior chapter. In brief, a transpalatal spring was anchored to pairs of left and right premolars across the palate were used to induce tooth movement over a five day period (**Figure 4**). Springs were tied via steel ligatures to bonded wires near the necks of the teeth, but not interfering with the gingiva. A force of 150 to 250 mg was placed over the four premolars during the loading of the spring. The spring was left in place and its load re-evaluated at each reading until its removal. With this model, there is a considerable tipping force, with the crown (above the center of resistance) being pulled lingually, and the root apices tipping buccally. In concept, the lingual portions of the tooth's sulcus would accumulate fluid of the adjacent regions of the PDL that are under compression, while the buccal portions would accumulate fluid from regions under tension. It is not evident how much mixing and flow of fluid occurs around the tooth's circumference, but four quadrants of the sulcus were sampled separately in order to test for possible differences (**Figure 7**).

Gingival Crevicular Fluid

Gingival crevicular fluid (GCF) normally accumulates from the gingival plexus of blood vessels in the gingival corium between the crevice of the attached gingiva and the tooth. The gingival corium lies subjacent to the epithelium lining the dentogingival space (Alfano 1974). Stress on the tooth, notably mechanical stress, up-regulates fluid expression (Meikle 2006; Wise and King 2008). These increases in response to mechanical stress are evident in **Figure 8**, which is a plot of the four lingual (compression) sites, and **Figure 9**, which is a plot of the four buccal (tension) sites.

The gingival crevice around the periphery of a tooth is continuous, so there should be mixing (and fluid homogenization) among the specific collection sites. The fluid volumes measured at all five examinations were summed to examine the correlation matrix of volumes (**Table 2**). All of the correlations were positive, and based on the sample size of 270 (and overlooking covariations among examinations), those correlations above $r = 0.13$ are statistically significant at alpha of 0.05. Correlations are low (*ca*. $r \approx 0.20$), suggesting a good deal of independence in fluid volumes around the crevicular circumference of the premolars.

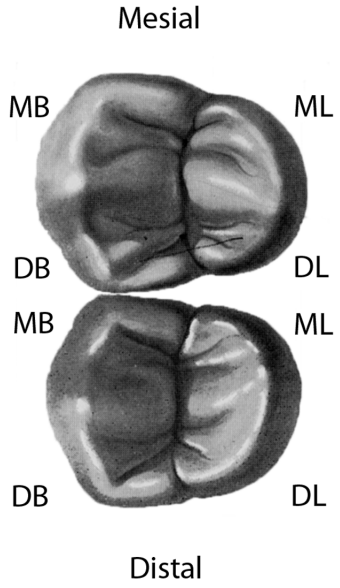

Figure 7. Schematic of the occlusal view of the maxillary right premolars. Crevicular fluid samples were obtained at each examination on a designated buccal and lingual location per tooth, mesiobuccal (MB), mesiolingual (ML), distobuccal (DB), and distolingual (DL). The same samples were collected from the two teeth in the left quadrant (not shown), and the left and right samples were combined for analysis. Eight paper strips were collected at each examination.

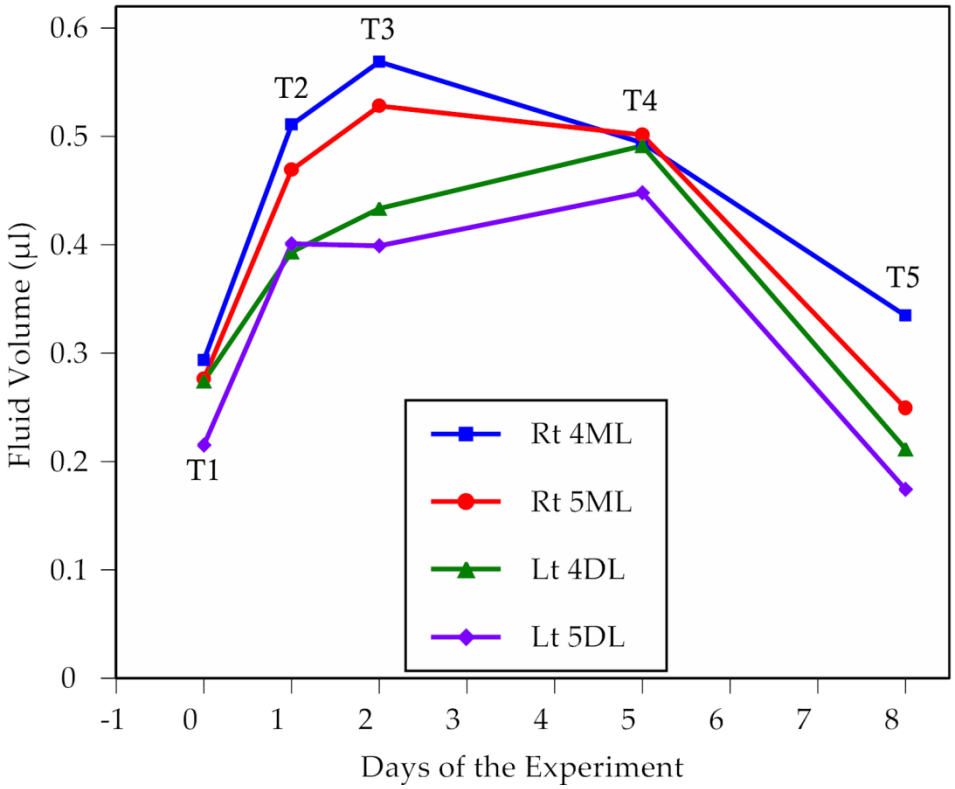

Figure 8. Plot of the amounts of crevicular fluid in the lingual quadrants of the crevice as a function of time.
The five examinations are scaled along the X axis. (T1 = day 0; T2 = day 1; T3 = day 2; T4 = day 5; T5 = day 8). Abbreviations are first premolar (tooth 4) and second premolar (5), left (Lt) and right (Rt), plus mesiolingual (ML), and distolingual (DL).

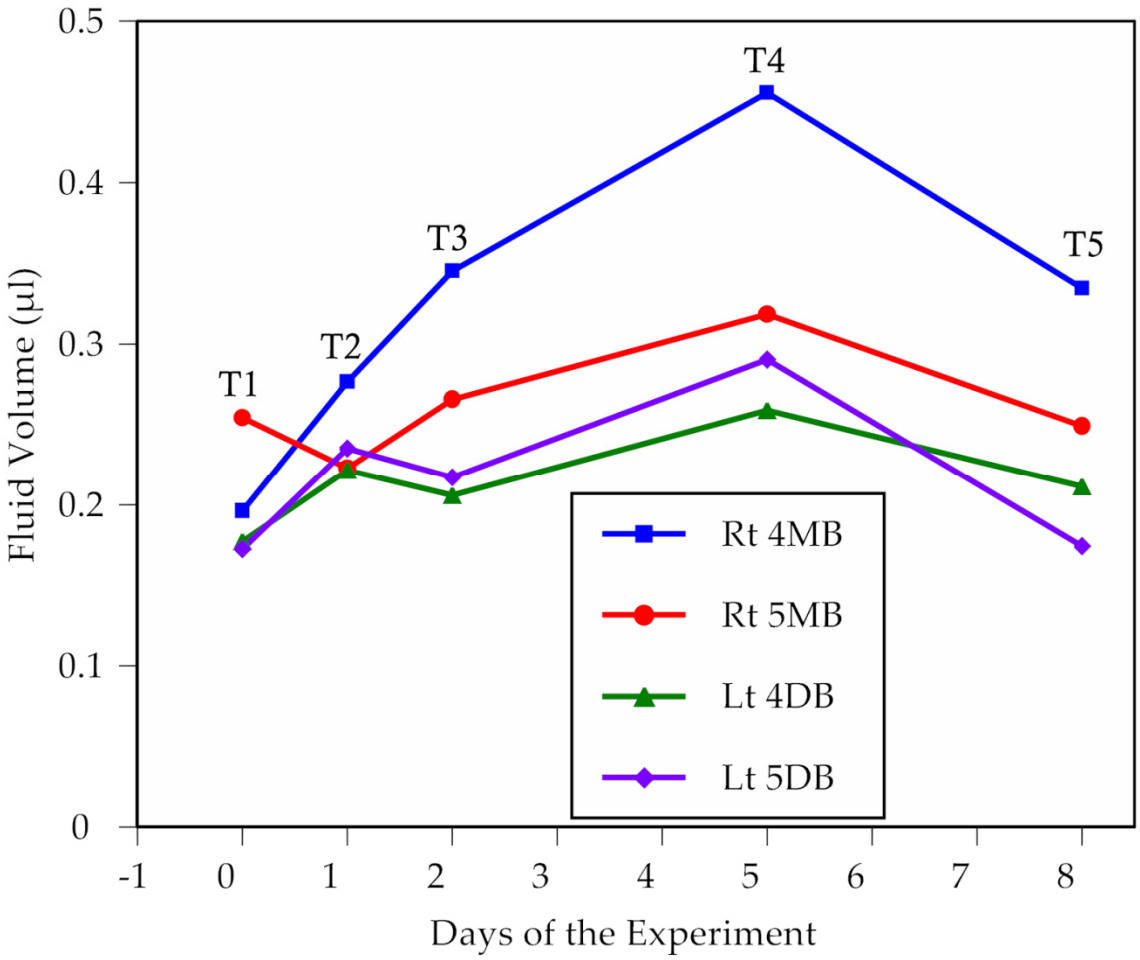

Figure 9. Plot of the amounts of crevicular fluid in the buccal quadrants of the crevice as a function of time.
The five examinations are scaled along the X axis. Abbreviations are first premolar (tooth 4) and second premolar (5), left (Lt) and right (Rt), plus mesiobuccal (MB), and distobuccal (DB).

Table 2. Matrix of correlations (n = 270) among the eight collection sites based on combining the crevicular fluid volumes at the five examinations.

Sites	R 4MB	R 5MB	L 4DB	L 5DB	R 4ML	R 5ML	L 4DL	L 5DL[1]
R 4MB	1.000	0.370	0.131	0.179	0.122	0.159	0.138	0.262
R 5MB	**0.370**	1.000	0.211	0.243	0.031	0.179	0.215	0.142
L 4DB	**0.131**	**0.211**	1.000	0.170	0.159	0.101	0.144	0.108
L 5DB	**0.179**	**0.243**	**0.170**	1.000	0.062	0.191	0.215	0.147
R 4ML	**0.122**	0.031	**0.159**	0.062	1.000	0.190	0.209	0.200
R 5ML	**0.159**	**0.179**	0.101	**0.191**	**0.190**	1.000	0.317	0.284
L 4DL	**0.138**	**0.215**	**0.144**	**0.215**	**0.209**	**0.317**	1.000	0.353
L 5DL	**0.262**	**0.142**	0.108	**0.147**	**0.200**	**0.284**	**0.353**	1.000

[1]The values of the five examinations were combined for these calculations. Values significant at ($P < 0.05$) in the lower-left half of the matrix are shown in bold and underlined. Abbreviations are left (L) and right (R) sides of the arch and mesiobuccal (MB), distobuccal (DB), mesiolingual (ML), and distolingual (DL) sites of fluid collection. The premolars are number as first (4) and second (5) premolar using Palmer notation.

Crevicular Fluid Side Differences

Analysis of the individual collection sites seemed unwarranted because of individual variability, but the sums of the buccal (tension) and lingual (compression) sides of the teeth were compared. **Table 3** (**Figure 10**) lists the results of paired t-tests at each of the five examinations. Of note, there was a statistically significant difference at each comparison, with the lingual volumes consistently greater than on the buccal sites. The correlations at each examination are positive but low, suggesting that the amounts of fluid on the two sides are largely independent. Since the fluid volumes are significantly greater on the buccal side at baseline, perhaps the side differences are due to anatomical differences rather than the nature of the force applied. **Figure 4** does suggest that the disparity between sides increased during the experiment (T2, T3, T4), and that the difference decreased by T5 after the spring had been removed.

Still, GCF volumes on both sides of the teeth behaved similarly: The smallest volumes were at baseline, and volumes increased duration of the spring (T2 through T4). The final readings were (T5) lower than at T2 through T4, but, at least lingually, had not returned completely to baseline. The lingual crevicular fluid level at day 0 was 1.09 (sd = 0.705) and at day 8 the reading was 1.39 (sd = 0.709), and, by paired t-test, the difference was marginally significant (t = 2.1; P = 0.043). The buccal crevicular fluid level at day 0 was 0.82 (sd = 0.570), and it was 0.97 (sd = 0.695) at day 8. This buccal difference was not significant (t = 1.28; P = 0.2060).

GCF levels increased following the baseline examination that was collected prior to spring placement. Visually, there was a sharper and higher rise for the lingual (compression side) readings than for the buccal samples. Also, the shapes of the curves were different; there was a more rapid rise on the lingual side, while the volume of the buccal samples did not reach their maxima until examination T4 (day5). Still, it is notable that the shapes of the curves, both for the buccal and lingual samples, are fairly similar given sampling fluctuations. There appears to be some covariation (correlation) among the sample sites, possibly because the total region is small (two adjacent teeth) and because of fluid flow and mixing within the crevice of each tooth.

Finally, it is evident in **Figures 8 and 9** that once the stressor (the spring) was removed after data collection at the T4 examination (day 5), the levels of crevicular fluid diminished. At examination T5 (3 days after spring removal), fluid levels had decreased to near the baseline level, but were still higher than before the spring was introduced.

Samples from all regions of the crevice were combined into the lingual and buccal portions, and the sum of these is the complete, circumferential total (**Figure 11**). This plot again shows that crevicular fluid is more common on the lingual than the buccal regions, even at the baseline examination, and that the rise in GCF caused by the spring is more rapid and greater on the lingual side. Again, it is unclear how dynamic

Table 3. Results of paired t-tests between the gingival fluid volumes collected from the buccal and lingual sides of the four maxillary premolars.

	Examination[1]				
Statistic	T1	T2	T3	T4	T5
Total Lingual	1.059	1.774	1.929	1.935	1.339
Total Buccal	0.800	0.956	1.034	1.324	0.347
Mean Difference	0.259	0.818	0.895	0.611	0.991
Std Error	0.105	0.128	0.125	0.116	0.097
Upper 95% CL	0.468	1.074	1.147	0.844	1.186
Lower 95% CL	0.050	0.562	0.644	0.378	0.796
n	58	53	53	53	53
Correlation	0.20	0.17	0.27	0.47	0.48
t-test	2.48	6.41	7.14	5.26	10.19
df	57	52	52	52	52
P-value	**0.0163**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**

Paired t-tests were performed at each of the five examinations.

[1]Statistically significant values are shown in bold font and underlined.

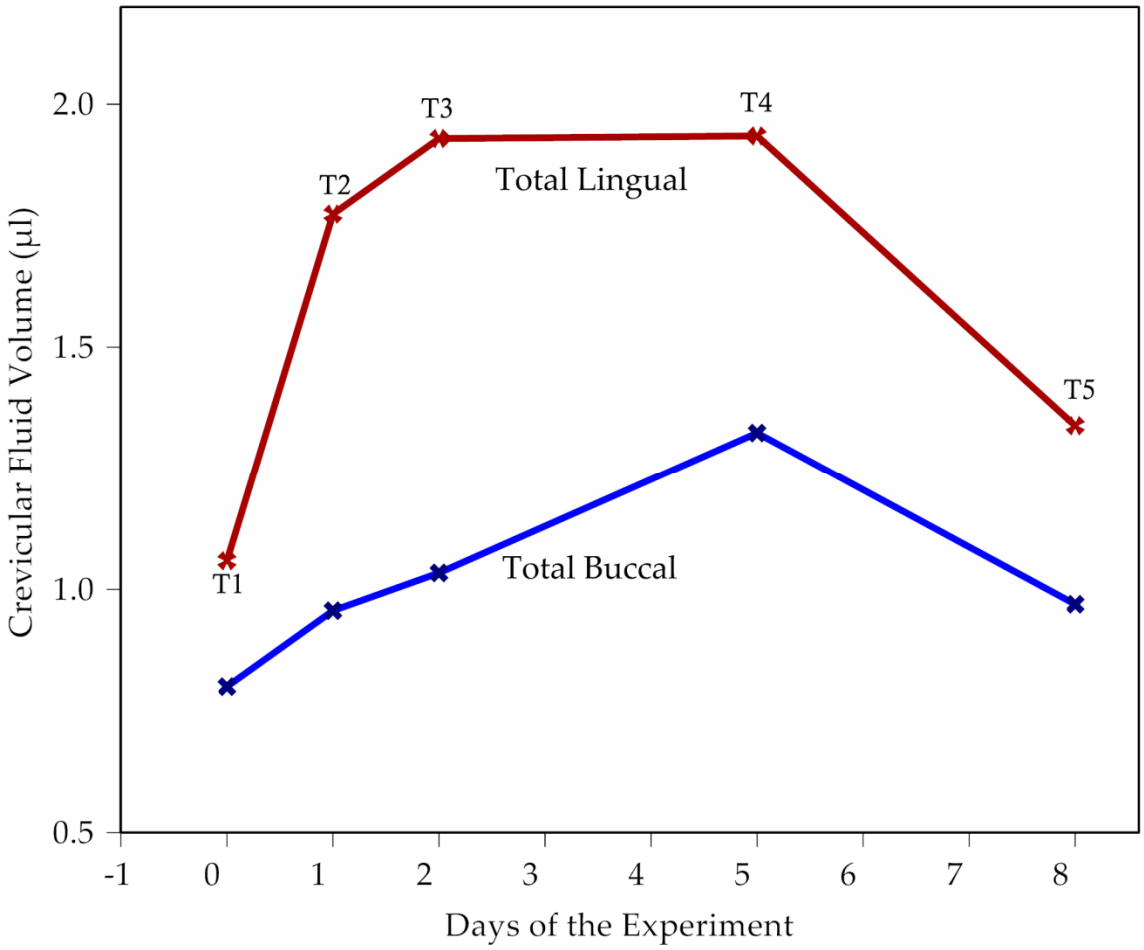

Figure 10. Plot of the buccal and lingual volumes of crevicular fluid across the five examinations.

The five examinations are scaled along the X axis to display the days of the experiment. Volumes on the lingual (compression) sides were consistently larger than those on the buccal. Both sides showed increased secretions after the spring was in place, and the volumes decreased when the stress was removed.

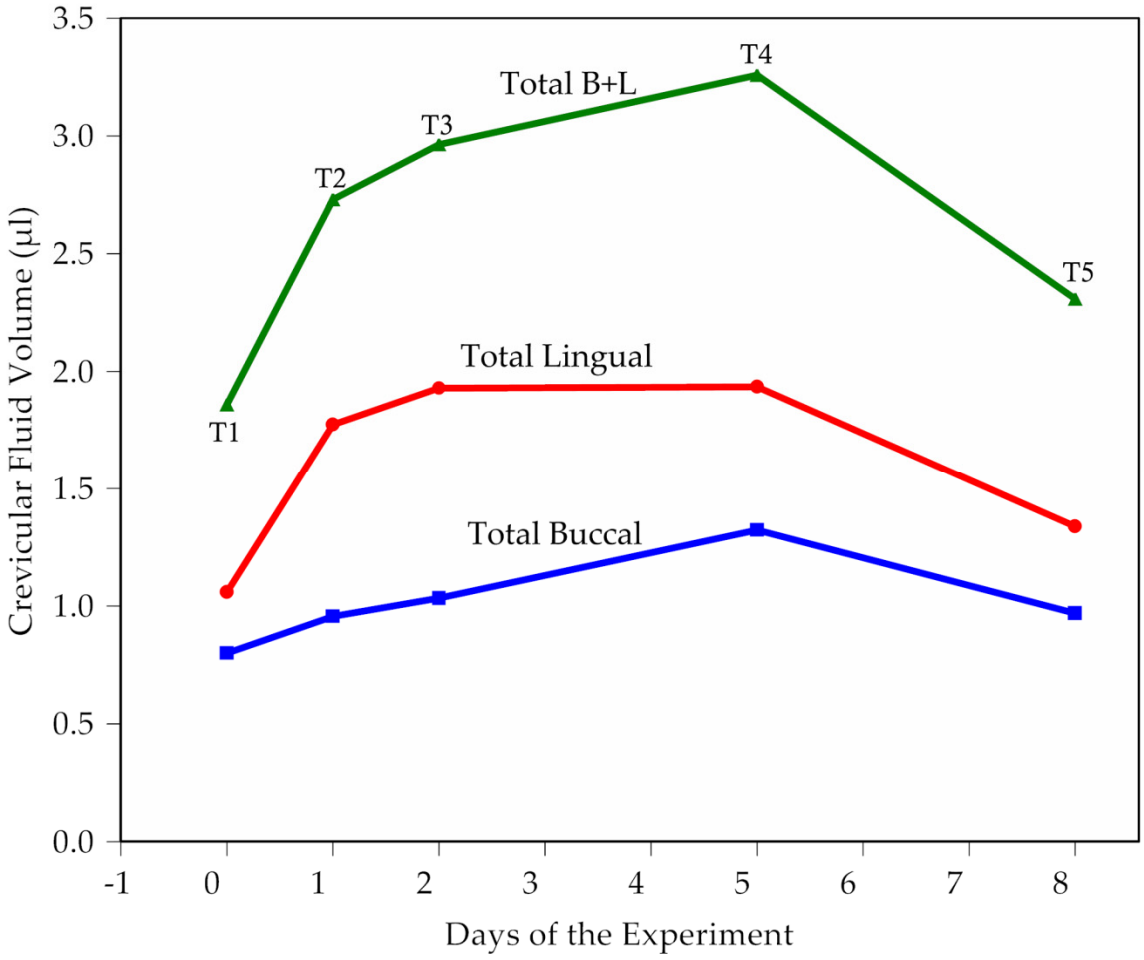

Figure 11. Plot of the volumes of crevicular fluid in the crevice as a function of site and time.
The five examinations are scaled along the X axis.

the fluid flow is around the crevice. It could be, for example, simply that there is more fluid on the lingual side because the anatomy of the structure causes it to accumulate there.

Crevicular Fluid: Statistical Examinations

The prior section described the visually-evident time changes, but the suggestive trends need to be confirmed statistically. A repeated-measures analysis of variance (MANOVA) model was used with the JMP version 9 statistical package (SAS Institute, Inc., Cary, North Carolina). The generalized linear model module in SPSS version 19 (IBM North America New York) also was used. The five examinations (time) were the repeated measure, and several dependent variables were tested.

Total Crevicular Fluid

As shown in **Figures 8 and 9**, there was some concordance in the time change from the samples collected from the eight sites between the two premolars. Consequently, rather than detailing the redundant statistical findings, analyses of three variables are emphasized, namely (1) the summed measures of the four buccal sites, (2) the summed of the four lingual sites, and (3) the total of all sites (**Figure 11**).

Buccal GCF Levels. Of course, this was a completely within-subjects statistical design. Examining the buccal means, the lowest amount of crevicular fluid (buccal sides) was invariably at the start of the experiment, prior to spring placement. For the sums (**Figure 12**), repeated-measures ANOVA yields a significant difference among examinations ($F = 4.55$; df = 4 and 49; $P = 0.0033$). The significant F-ratio indicates that there was at least one statistically significant difference among the means, but it does not say where the differences were located. Contrasts (**Table 2**) were used to test for differences between the examinations; this method compares each examination to the initial examination (*e.g.*, Y2-Y1, Y3-Y1, Y4-Y1, and Y5-Y1). This is an option in JMP, and it is practical in that each change is compared back to the baseline (pretreatment) condition. A complementary approach is to test the differences pairwise using paired t-tests (**Table 3**).

Using this approach for the buccal samples (**Table 4**), fluid levels were elevated at examination T2 compared to baseline, but not significantly so ($P = 0.0547$). Likewise, the average level at examination T3 was absolutely elevated, but not significantly so compared to baseline. Then there was a substantial increase by examination T4, which levels were increased by this duration of treatment. And then, by examination T5, which was three days after spring removal, levels had returned to a level not significantly different from baseline ($P = 0.2061$). The interval between examinations T4

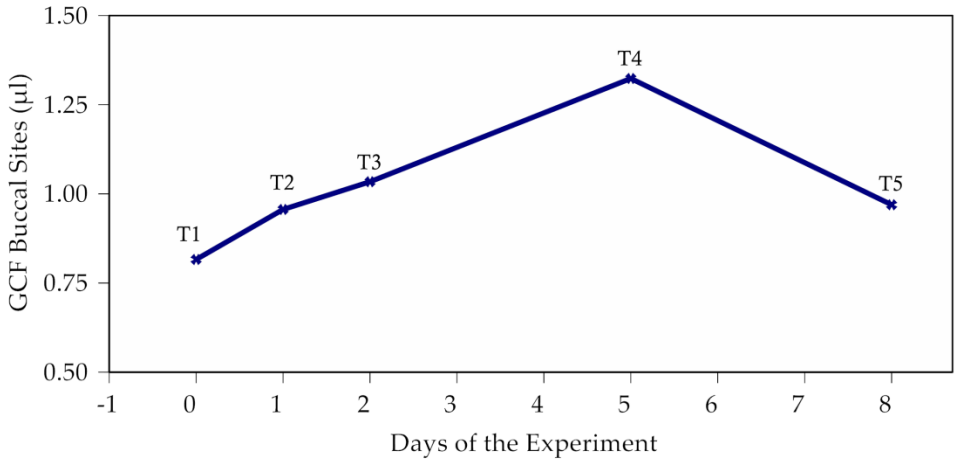

Figure 12. Plot of the volumes of buccal crevicular fluid across the five examinations.
The five examinations are scaled along the X axis to represent the days of the experiment.

Table 4. Results of contrast tests for the buccal volumes of crevicular fluid.

Comparison	df	F-ratio	P-value
1 *vs.* 2	1, 52	1.90	0.1738
1 *vs.* 3	1, 52	3.86	0.0547
1 *vs.* 4	1, 52	16.55	**0.0002**
1 *vs.* 5	1, 52	1.64	0.2061

and T5 was three days, which shows that the return to baseline was fairly rapid.

Table 4 (contrasts) is complemented by the matrix of paired t-tests (**Table 5**), which further shows that the source of statistical significance involves T4 alone. That is, the single source of significance was between T4 and the four other examinations. Possibilities are that T4 is simply aberrant or that, because it is the longest exposure to the spring, fluid levels are being up-regulated because it took that duration of time to initiate some processes of tooth movement.

Lingual GCF Levels. Figure 11 also plots the sample means for the lingual samples. By repeated-measures ANOVA, there was a highly significant change in fluid quantities among examinations ($F = 12.09$; df = 4 and 49; $P < 0.0001$). Contrasts for this variable (**Table 6**) show that quantities rose considerably from examinations T1 to T2 ($P < 0.0001$), and continued to increase through examination T3. The highest mean level **(Figure 13)** was achieved at examination T4, which was the end of spring-treatment. Thereafter, following spring removal, levels dropped by examination T5 so that levels were barely above the baseline level ($P = 0.0434$). **Table 7** (matrix of P-values from paired t-tests) complements the contrasts, emphasizing the significant rise from T1 to T2-T3-T4, and then a significant drop in fluid volumes once the spring was removed.

Total GCF Levels. Changes in total GCF volume are predictable since they are simply the sums of the prior two volumes just examined (**Figure 14**). Changes across examinations were highly significant by longitudinal analysis ($F = 10.72$; df = 4 and 49; $P < 0.0001$). One day of spring-wear significantly elevated fluid volume, and the mean was further elevated by day 2 (examination T3). The highest mean was for examination T4, which was five days of spring wear. The table of paired t-tests (**Table 8**) shows that the source of significance were two-fold, namely (1) a significant increase in volume between T1 and T2 when the spring was placed and (2) a significant decrease between T4 and T5 when the spring was removed. **Table 9** lists the contrasts. There was a maximum GCF volume at T4, and that also was the longest the spring was in place. It is

Table 5. Matrix of P-values from paired t-tests to interpret the source of significance of the MANOVA test for GCF from the buccal sites across the five examinations.

	Group[1]			
Group	T1	T2	T3	T4
T2	0.1738			
T3	0.0547	0.4485		
T4	**0.0002**	**0.0038**	**0.0132**	
T5	0.2060	0.9107	0.5686	**0.0015**

[1]Statistically significant values are shown in bold font and underlined.

Table 6. Results of contrast tests for the lingual volumes of crevicular fluid.

Comparison	df	F-ratio	P-value
1 *vs.* 2	1, 52	32.24	**< 0.0001**
1 *vs.* 3	1, 52	31.25	**< 0.0001**
1 *vs.* 4	1, 52	34.66	**< 0.0001**
1 *vs.* 5	1, 52	4.29	**0.0434**

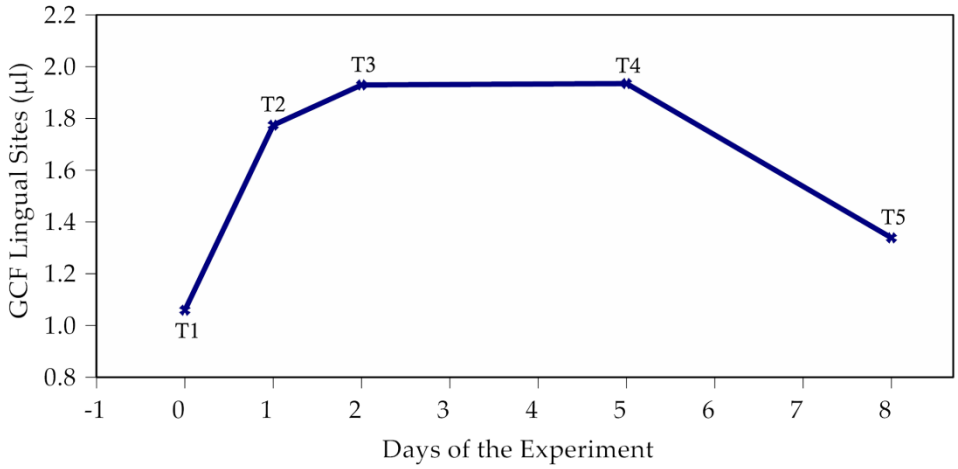

Figure 13. Plot of least-squares means from MANOVA for lingual gingival crevicular fluid.
The five examinations are scaled along the X axis to show days of the experiment. The overall difference among examinations is highly significant ($P < 0.0001$).

Table 7. Matrix of P-values from paired t-tests to interpret the source of significance of the MANOVA test for GCF from the lingual sites across the five examinations.

	Group[1]			
Group	T1	T2	T3	T4
T2	**< 0.0001**			
T3	**< 0.0001**	0.2640		
T4	**< 0.0001**	0.2551	0.9684	
T5	**0.0434**	**0.0019**	**< 0.0001**	**0.0003**

[1]Statistically significant values are shown in bold font and underlined.

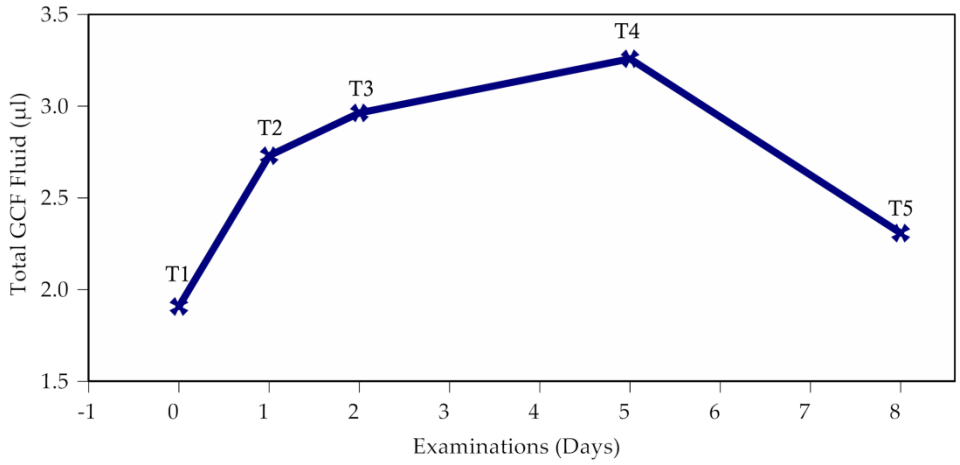

Figure 14. Plot of least-squares means from MANOVA for total gingival crevicular fluid.
The five examinations are scaled along the X axis to show days of the experiment. The differences among examinations were highly significant ($P < 0.0001$).

Table 8. Matrix of P-values from paired t-tests to interpret the source of significance of the MANOVA test for GCF sum of sites across the five examinations.

	Group[1]			
Group	T1	T2	T3	T4
T2	**< 0.0001**			
T3	**< 0.0001**	0.2083		
T4	**< 0.0001**	**0.0136**	0.1500	
T5	**0.0426**	**0.0294**	**0.0013**	**< 0.0001**

[1]Statistically significant values are shown in bold font and underlined.

Table 9. Results of contrast tests for the total volumes of crevicular fluid.

Comparison	df	F-ratio	P-value
1 *vs.* 2	1, 52	10.72	**< 0.0001**
1 *vs.* 3	1, 52	25.01	**< 0.0001**
1 *vs.* 4	1, 52	33.82	**< 0.0001**
1 *vs.* 5	1, 52	4.31	**0.0426**

unknown when a physiological maximum of fluid could be obtained because the fluid quantity increased monotonically until the spring was removed at day 5, because there was no long-term data. While total volume decreased significantly from T4 to T5, the value at T5 was still noticeably above the baseline level (P = 0.0426; **Table 9**). An interesting detail would be to determine when the fluid levels actually returned to baseline after five days of spring treatment. Likewise, in an actual orthodontic setting, how long after appliance placement does it take before fluid levels return to normal? And, do they increase discernibly at each appointment when new forces are initiated?

Fluid quantities dropped rapidly from day T4 to T5, and the final value was only slightly elevated compared to the baseline (P = 0.0426). This reiterates the prior findings that GCF quantities respond quickly to the mechanical stress of a spring, and about as quickly to its removal. It also raises the question of how fluid levels respond to the force-dissipation of arch wires and other appliances between appointments.

Effectors of Fluid Volumes

The analyses above deal with the total sample of subjects, but an aim of this project was to test for age, sex, and race differences. That is, does the age, or sex, and/or race of a patient systematically influence his fluid volume response? Repeated-measures MANOVA models again were used to test for these possibilities. A three-way model (plus time) was used initially; it was a mixed model since race, sex, and age category were fixed effects, while the five examinations (time) was a repeated measure.

MANOVA results for the data from the buccal sites are listed in **Table 10**. Time, as before, was highly significant because fluid levels were responsive to the mechanical stress of the spring. None of the other effects was significant. By this measure, neither race, sex, nor age of the patient had a systematic influence on the amount of crevicular GCF produced.

Table 11 lists the statistical results for the fluid volumes from the lingual sites. There were highly significant differences among the five examinations, but race, sex, and age played no discernible role in influencing fluid levels. Race and sex carry very high P-values, close to one, so it seems unlikely that either factor influenced fluid levels. However, age—whether the subject was an adolescent or adult—had a non-significant but low probability (P = 0.09). Plotting the mean fluid levels by age category, showed that adults had the larger mean at all five examinations (**Figure 15**). The age difference did not achieve significance (P = 0.09) because of intra-group variability, but the results are suggestive, and further work with a larger and more homogeneous sample is indicated.

Table 10. MANOVA results for crevicular fluid measured from the buccal sites.

Source	F-ratio	df	P-value[1]
Between Subjects			
Race	2.71	1, 45	0.1066
Sex	2.32	1, 45	0.1345
Age	0.61	1, 45	0.4400
Race-x-Sex	0.61	1, 45	0.4375
Race-x-Age	0.49	1, 45	0.4872
Sex-x-Age	2.94	1, 45	0.0933
Race-x-Sex-x-Age	1.31	1, 45	0.2579
Within Subjects			
Time	4.13	4, 42	**0.0065**
Time-x-Race	1.32	4, 42	0.2772
Time-x-Sex	1.61	4, 42	0.1901
Time-x-Age	1.02	4, 42	0.4106
Time-x-Race-x-Sex	1.88	4, 42	0.1315
Time-x-Race-x-Age	0.74	4, 42	0.5698
Time-x-Sex-x-Age	0.91	4, 42	0.4653
T-x-R-x-S-x-A	2.11	4, 42	0.0959

[1]Statistically significant values are shown in bold font and underlined.

Table 11. MANOVA results for crevicular fluid measured from the lingual sites.

Source	F-ratio	df	P-value[1]
Between Subjects			
Race	0.00	1, 45	0.9644
Sex	0.00	1, 45	0.9954
Age	3.00	1, 45	0.0900
Race-x-Sex	0.03	1, 45	0.8629
Race-x-Age	0.23	1, 45	0.6376
Sex-x-Age	0.82	1, 45	0.3706
Race-x-Sex-x-Age	0.16	1, 45	0.6921
Within Subjects			
Time	12.42	4, 42	**< 0.0001**
Time-x-Race	0.73	4, 42	0.5794
Time-x-Sex	0.37	4, 42	0.8288
Time-x-Age	1.26	4, 42	0.2992
Time-x-Race-x-Sex	1.10	4, 42	0.3701
Time-x-Race-x-Age	0.94	4, 42	0.4487
Time-x-Sex-x-Age	0.79	4, 42	0.5381
T-x-R-x-S-x-A	0.68	4, 42	0.6070

[1]Statistically significant values are shown in bold font and underlined.

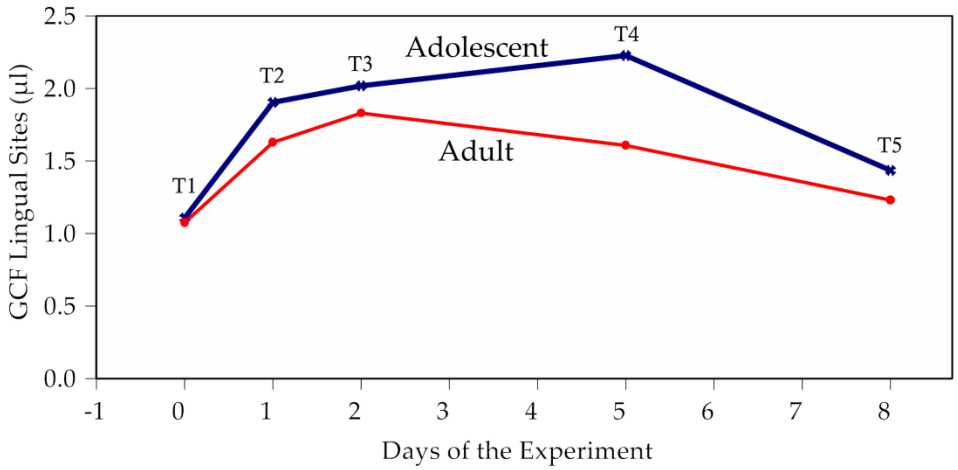

Figure 15. Plot of the lingual GCF levels for each examination, by age category.
Though statistically not significant (P = 0.0526) by MANOVA, adolescents consistently exhibited higher fluid concentrations than adults across the five examinations.

MANOVA analysis for the total crevicular fluid samples is shown in **Table 12**. Race and sex were, again, far from significant, and, statistically, the probability for the age category is no longer suggestive (P = 0.46). Since "total fluid" is the summation of the individual's fluid responses (the total of all samples), it is discussed in more detail.

The variable 'race' was consistently non-significant (**Figure 16**). Fluid levels did not differ between these samples of American blacks and whites (P = 0.3880). As for sex of the patient, there is no suggestion of a difference (**Figure 17**; P = 0.4376). The difference between the two age category were visually suggestive of adults having higher levels, but not significantly so (**Figure 18**; P = 0.4624).

In sum, the crevicular fluid levels were tested for differences between races (American black-white), sexes, and age category (child or adult). Tested with MANOVA, there was no systematic difference in GCF volumes for any of these variables. Notably, this three-way model accounts for sampling inequalities in sample size among race, sex, and age (*i.e.*, an unbalanced design). This is a parsimonious, objective solution that is superior (and different) from univariate analysis, where one variable does not control for the other two. Consistently in these analyses 'time'—a proxy for the duration of tension on the teeth by the transpalatal spring—was highly significant. Force from the spring significantly increased the fluid levels on both the buccal and lingual sides of the tooth. The longer the spring was in place (a total of 5 days), the greater the GCF levels. It is not known because of this short interval when the maxima might be reached in actual

Table 12. MANOVA results for crevicular fluid measured from the total of all sites.

Source	F-ratio	df	P-value
Between Subjects			
Race	0.76	1, 45	0.3880
Sex	0.61	1, 45	0.4376
Age	0.55	1, 45	0.4624
Race-x-Sex	0.27	1, 45	0.6092
Race-x-Age	0.45	1, 45	0.5062
Sex-x-Age	2.17	1, 45	0.1481
Race-x-Sex-x-Age	0.10	1, 45	0.7475
Within Subjects			
Time	11.26	4, 42	**< 0.0001**
Time-x-Race	1.21	4, 42	0.3224
Time-x-Sex	0.81	4, 42	0.5259
Time-x-Age	1.34	4, 42	0.2695
Time-x-Race-x-Sex	1.72	4, 42	0.1645
Time-x-Race-x-Age	0.95	4, 42	0.4472
Time-x-Sex-x-Age	0.66	4, 42	0.6254
Time-x-Race-x-Sex-x-Age	1.58	4, 42	0.1960

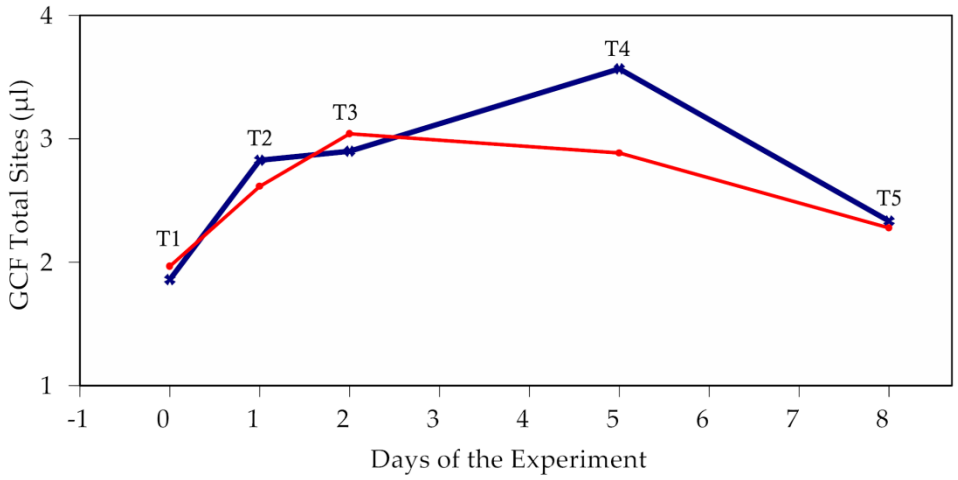

Figure 16. Graph of the crevicular fluid for the total of all sites, by examination and race.
The five examinations are scaled along the X axis to show days of the experiment. There was no suggestion of any black-white race difference in fluid levels.

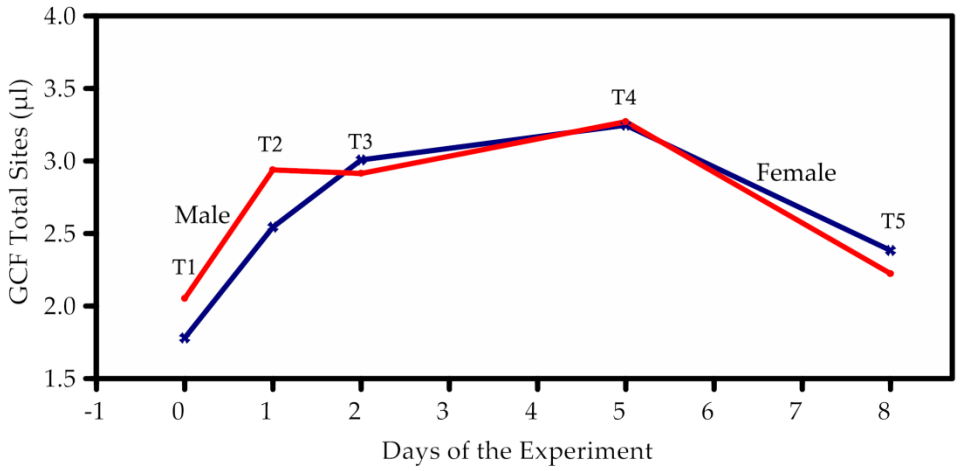

Figure 17. Graph of the crevicular fluid for the total of all sites, by examination and sex.
There was no suggestion of any male-female difference in GCF volumes.

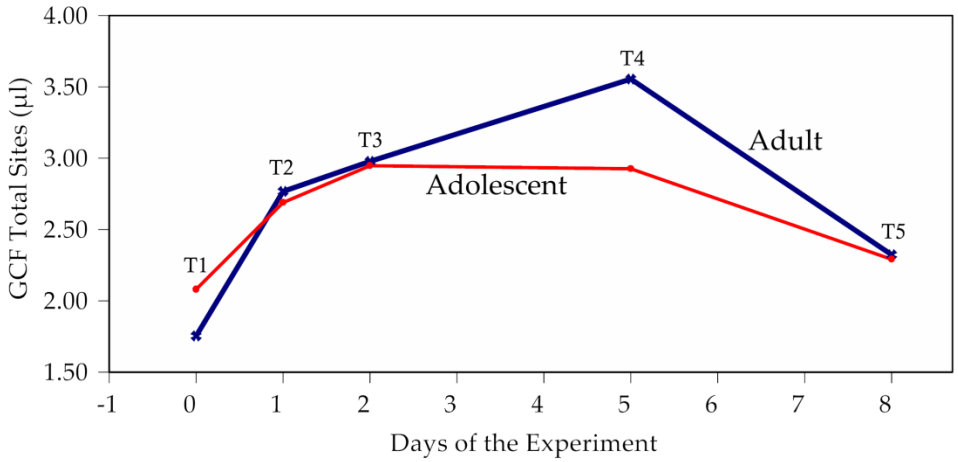

Figure 18. Graph of the crevicular fluid for the total of all sites, by examination and age category.
There was a suggestion in these means for adults to have higher fluid levels, but, statistically, there was no difference (P = 0.4624).

orthodontic settings. Once the force was removed—the spring was removed at day 5—the fluid levels diminished. By three days after spring removal (day 8), fluid levels were close to, but slightly above the baseline values.

The next consideration addressed was whether the buccal and lingual collection sites were different enough that pooling the samples obscured age, sex, or race differences. For completeness, a series of MANOVA tests was calculated on the sides of the teeth separately. The first set of tests was for the four buccal sites alone.

GCF Collection Sites

As a starting point, the nature of the distribution of GCF volumes for the total (B+L) collection sites was assessed (**Figure 19**). This graph plots the individual data for each of the longitudinal study, but the times between examinations is constant.

The GCF volumes from the buccal and lingual sites (B + L = total) are fairly concordant. The GCF volumes from the buccal sites (**Figure 20**) were tested for a difference among examinations. Results of the MANOVA test (**Table 13**) show that time was indeed statistically significant (F = 4.55; df = 4 and 49; P = 0.0033). The table of paired t-tests (**Table 14**) disclosed that fluid levels were lowest at baseline and increased through T2, T3, and T4. The highest levels (T4) occurred when the spring was in place for the longest period of time. Statistically, T1 and T2 were the same, but T3 was

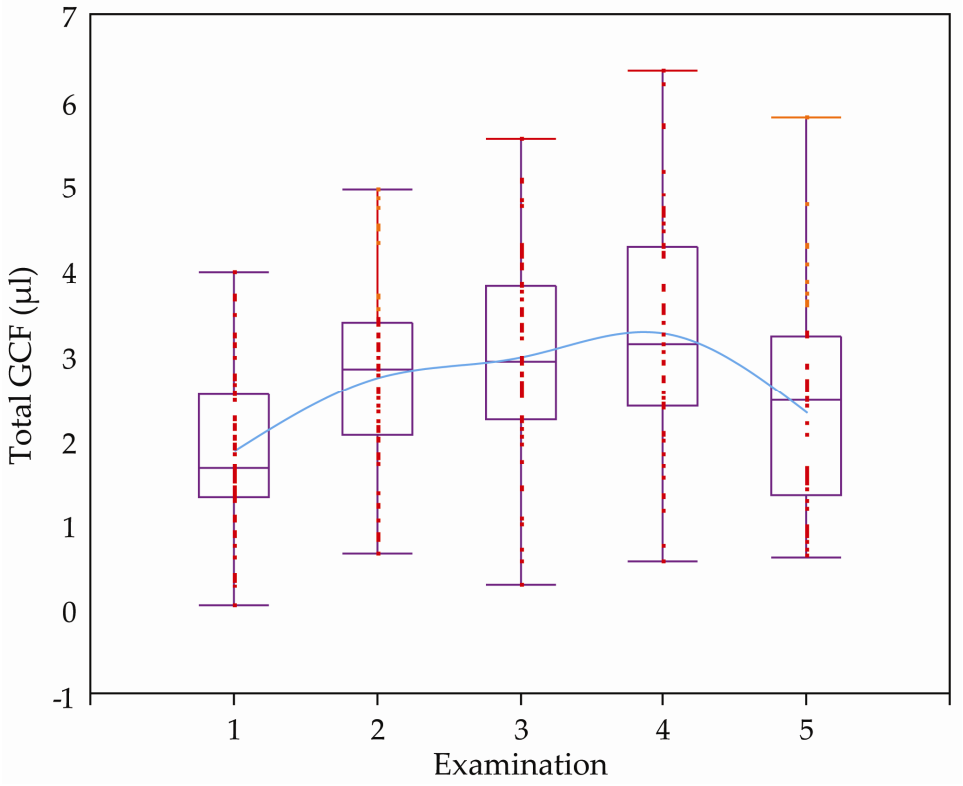

Figure 19. Boxplots of the volumes of total GCF.
There is a rise in volume by examination 3 that becomes greater by examination 4, but these appear to be leveraged by a few, large outliers. The shape of the boxplots shows that the distributions tend to be positively skewed.

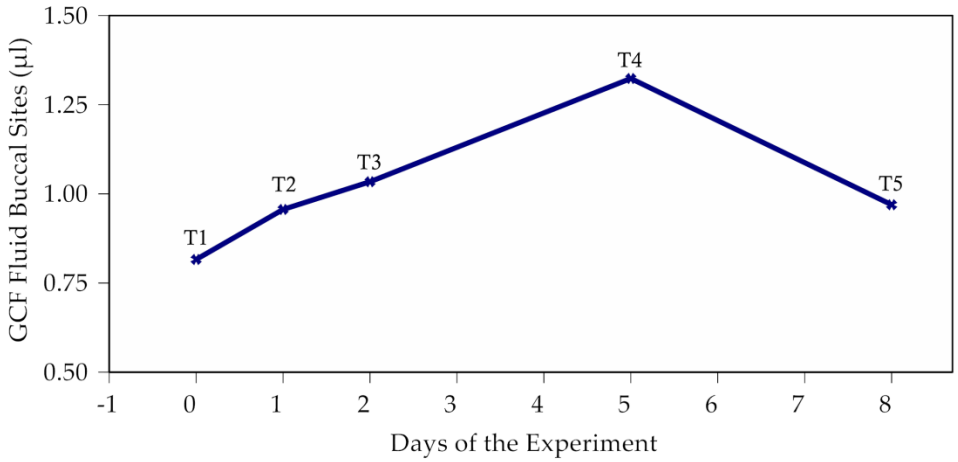

Figure 20. Plot of the volumes of GCF from the buccal sites.
The least-squares means are plotted for the five examinations. The volumes rose to a high by T4, when the spring was removed, then dropped to near-baseline by the final reading at T5.

Table 13. MANOVA results testing for age difference in the GCF fluid volumes on the buccal sites over the five examinations.

Source	F-ratio	df	P-value
Between Subjects			
Age Category	2.55	1, 51	0.1167
Within Subjects			
Time	4.45	4, 48	**0.0038**
Time-x-Age	0.55	4, 48	0.7026

Table 14. Matrix of P-values from paired t-tests to interpret the source of significance of the MANOVA test for GCF on the buccal sites across the five examinations.

	Group			
Group	T1	T2	T3	T4
T2	0.1738			
T3	0.0547	0.4485		
T4	**0.0002**	**0.0038**	**0.0132**	
T5	0.2060	0.9107	0.5686	**0.0015**

significantly higher than T1. Then there was a significant drop in volume between T4 and T5 (P = 0.0015), such that the volume at T5 was not significantly different than at baseline (P = 0.2060).

Lingual GCF Volumes. The natures of the distribution of GCF volumes across the course of the experiment are shown in **Figure 21**.

The plot for the lingual sites alone is shown in **Figure 22**, where there also was a significant difference across examinations (F = 12.09; df = 4 and 49; P < 0.0001). The source of significance is interpreted from the table of paired t-tests (**Table 15**). There was a significant increase in fluid from T1 to T2 (P < 0.0001), and the volumes stayed elevated from T2 through T4, then they dropped after spring removal, but the level at T5 was still significant higher than at baseline (P = 0.0434).

Lingual GCF Volumes: Age Test. The MANOVA test for a difference between age categories for the lingual volumes of GCF was not significant but suggestively low (P = 0.0526). The pattern of the data is shown in **Figure 23**, and the statistical results are listed in **Table 16**.

Lingual GCF Volumes: Sex Test. **Figure 24** shows the pattern of lingual fluid levels between the sexes, and females tend to have the larger volumes, but not significantly so as shown in **Table 17** (P = 0.4031). The lack of significance for 'sex' suggests that there was considerable variation within the groups.

Lingual GCF Volumes: Race Test. Volumes on the lingual sites were compared between races (American blacks and whites). The patterns are very similar between the two races (**Figure 25**), and these two groups were not statistically different by MANOVA (**Table 18)** (P = 0.9460).

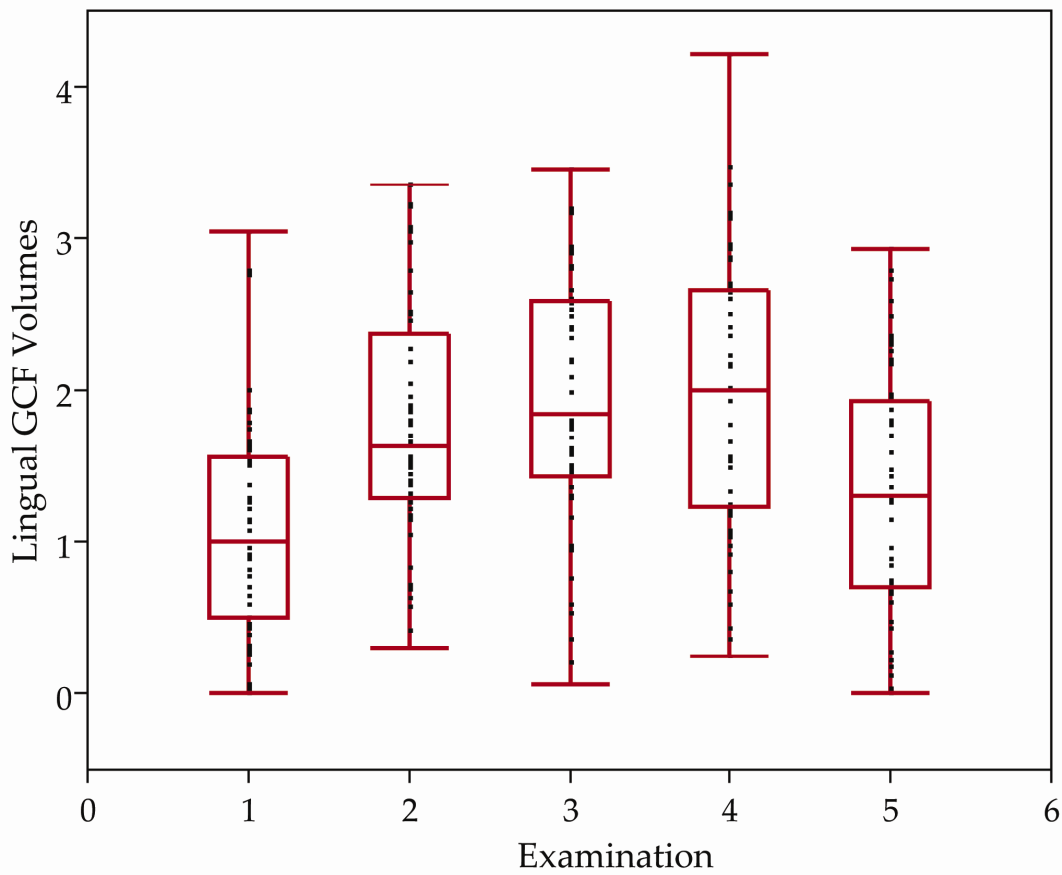

Figure 21. Boxplots of the volumes of GCF at the lingual sites across the five examinations.
There is a noticeable rise from examination 1 through 4, and then a decrease at examination 4 after the transpalatal spring was removed at examination 4.

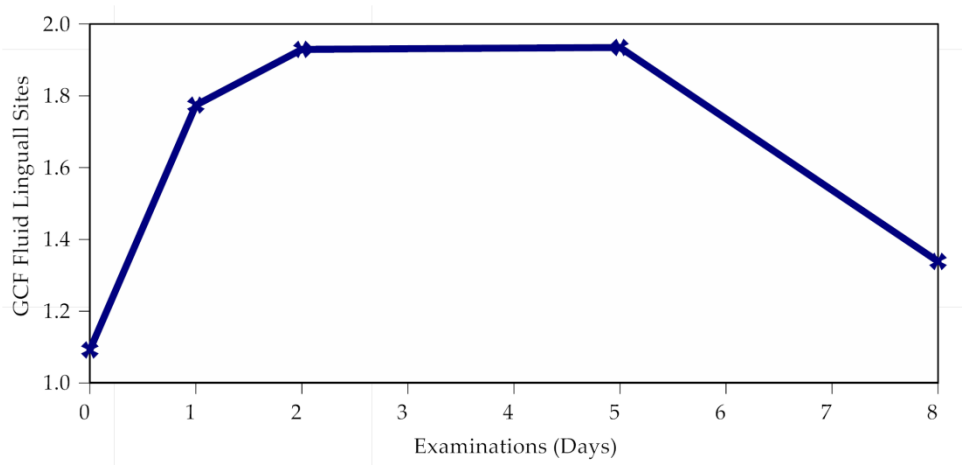

Figure 22. Plot of the volumes of GCF from the lingual sites.
The least-squares means are plotted for the five examinations (T1 = day 0, T2 = day 1, T3 = day 2, T4 = day5, and T5 = day 8). The MANOVA result was statistically significant ($P < 0.0001$) for differences across time.

Table 15. Matrix of P-values to interpret the source of significance of the MANOVA test for GCF on the lingual sites across the five examinations.

	Group			
Group	T1	T2	T3	T4
T2	**< 0.0001**			
T3	**< 0.0001**	0.2640		
T4	**< 0.0001**	0.2551	0.9684	
T5	**0.0434**	**0.0019**	**< 0.0001**	**0.0003**

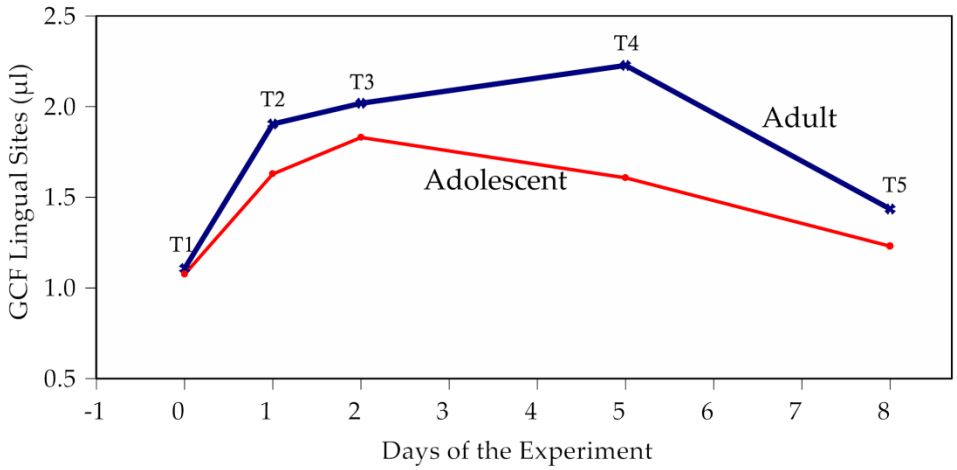

Figure 23. Plot of the volumes of GCF from the lingual sites by age.
The least-squares means are plotted for the five examinations. The MANOVA result was strictly not statistically significant (P = 0.0526), though the least-squares means tend to be higher in adults than adolescents.

Table 16. MANOVA results testing for age difference in the GCF fluid volumes on the lingual sites over the five examinations.

Source	F-ratio	df	P-value[1]
Between Subjects			
Age Category	3.94	1, 51	0.0526
Within Subjects			
Time	12.03	4, 48	< **0.0001**
Time-x-Age Category	1.33	4, 48	0.2708

[1]Statistically significant values are shown in bold font and underlined.

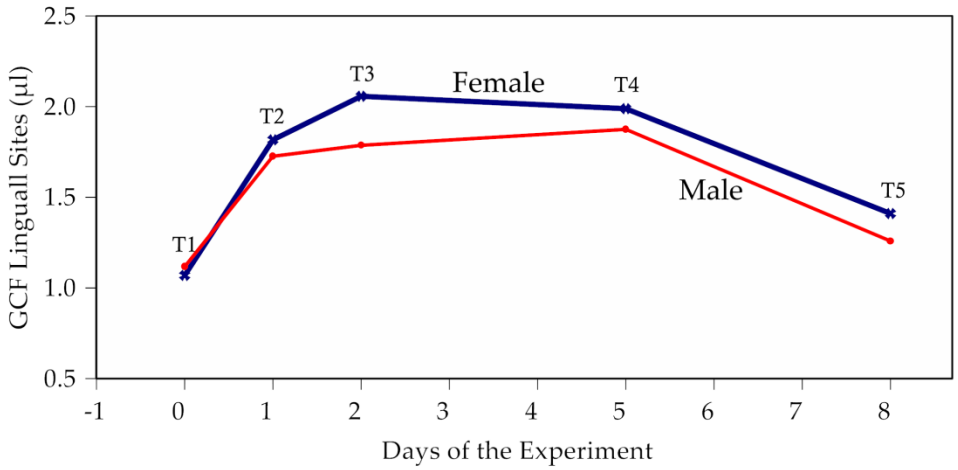

Figure 24. Plot of the volumes of GCF from the lingual sites by sex.
The least-squares means are plotted for the five examinations. The MANOVA result was not statistically significant (P = 0.4031), though the least-squares means tended to be higher in females than males.

Table 17. MANOVA results of testing for sex difference in the GCF fluid volumes on the lingual sites across the five examinations.

Source	F-ratio	df	P-value
Between Subjects			
Sex	0.71	1, 51	0.4031
Within Subjects			
Time	11.80	4, 48	**< 0.0001**
Time-x-Sex	0.02	4, 48	0.8805

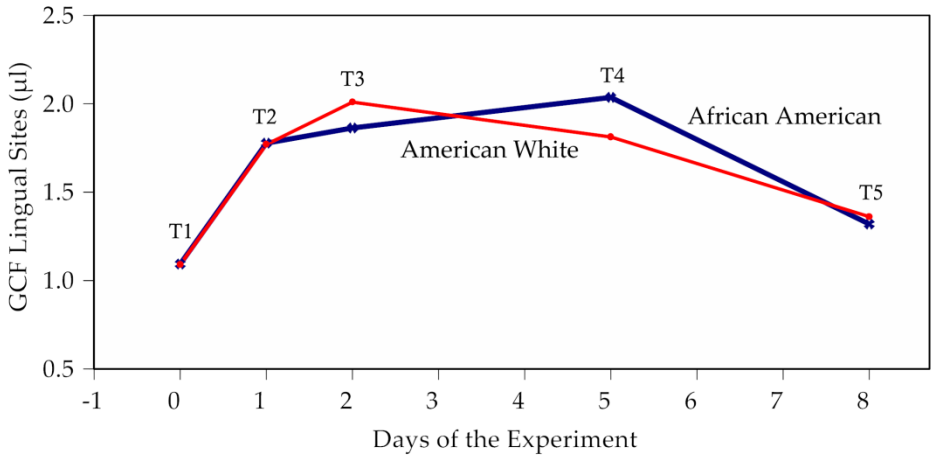

Figure 25. Plot of the volumes of GCF from the lingual sites by race.
The least-squares means are plotted for the five examinations. The MANOVA result was not statistically significant (P = 0.9460).

Table 18. MANOVA results testing for race difference in the GCF fluid volumes on the lingual sites across the five examinations.

Source	F-ratio	df	P-value[1]
Between Subjects			
Race	0.00	1, 51	0.9460
Within Subjects			
Time	11.69	4, 48	**< 0.0001**
Time-x-Race	0.46	4, 48	0.7657

[1]Statistically significant values are shown in bold font and underlined.

Figure 26 is a boxplot of the GCF lingual volumes between these blacks and whites (examinations pooled) just to confirm the considerable overlap between these two races.

Buccal GCF Volumes. The tests just reviewed for the GCF volumes collected from the lingual tooth sites are now duplicated for the buccal sites. The overall result, with no dependent variables, is shown in **Figure 27**. Statistically, the changes across time were significant statistically (P = 0.0033). The table of paired t-tests (**Table 19**) disclosed that the single source of significance was due to the maximum level seen on day 5 (T4); the first three samples (T1, T2, T3) were significantly lower than that at T4. As noted previously, T4 is the longest time the spring was in place, so this maximum may represent the progressive rise in fluid level prior to removal of the spring.

Buccal GCF Volumes: Age Test. Results of testing for an age difference in the GCF volumes collected from the buccal sites are shown in **Table 20**. There was not a significant difference between the age grades (P =0.1167), but inspection of the LS means (**Figure 28**) suggests that adolescents tended to have larger volumes than the adults.

Buccal GCF Volumes: Sex Test. The pattern for GCF volumes across the five examinations is graphed in **Figure 29**, and the noticeable difference is that males have the larger volumes according to these LS means. Results of the MANOVA test (**Table 21**) showed that the fluid levels did not differ significantly between the sexes, but the difference (P = 0.0686) is suggestive, with males having the larger volumes, even at baseline prior to treatment

Buccal GCF Volumes: Race Test. Was there a significant difference between American blacks and whites in the volumes of buccal GCF volume? The race difference is graphed in **Figure 30**, and the MANOVA results are listed in **Table 22**. The figure shows that the volumes were not consistently greater in either group. The race difference was not significant (P = 0.2475).

OPG and RANKL Concentrations

A driving issue in this study was to examine the nature of the distribution of RANKL and OPG. There was sufficient gingival crevicular fluid to make two assays from each sample (detailed below), and the results described below (**Figures 31** and **32**) were based on the arithmetic average of these duplicate measures. Some of these averages have concentrations below that detectable by the assay (concentrations not "zero," but too low to register), most concentrations for RANKL (**Figure 31**), were in the range of 0 to 100, and a few have much higher concentrations. Decidedly, the distribution is positively skewed. For RANKL, skewness was 4.99 and kurtosis was 37.17—both measures deviate substantially from a Gaussian distribution. Most of the

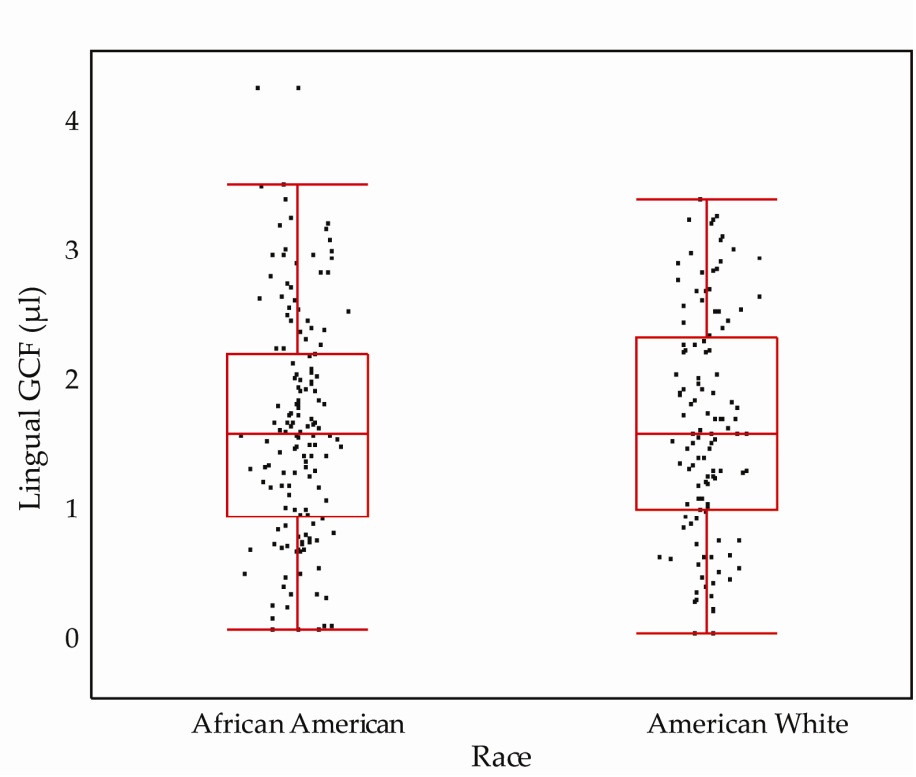

Figure 26. Boxplots of the GCF volumes collected from the lingual sites.
There is considerable overlap in the distributions, which accounts for the absence of any sort of statistical difference between the races.

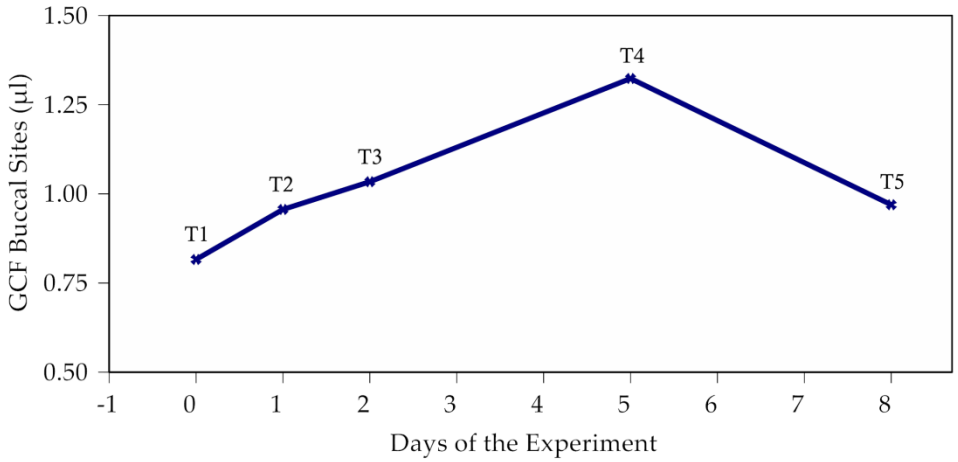

Figure 27. Plot of the volumes of GCF from the buccal sites of the premolars. The least-squares means are plotted for the five examinations. There is a rise in fluid till day 5 when the spring was removed, then a significant drop.

Table 19. Matrix of P-values from paired t-tests to interpret the source of significance of the MANOVA test for GCF on the buccal sites across the five examinations.

	Group			
Group	T1	T2	T3	T4
T2	0.1738			
T3	0.0547	0.4485		
T4	**0.0002**	**0.0038**	**0.0132**	
T5	0.2060	0.9107	0.5686	**0.0015**

Table 20. Results of MANOVA testing for a difference in concentrations of GCF from the buccal sites depending on the subject's age category.

Test	F-ratio	df	P-value[1]
Between Subjects			
Age Category	2.55	1, 51	0.1167
Within Subjects			
Time	4.45	4, 48	**0.0038**
Time-x-Age Category	0.55	4, 48	0.7026

[1]Statistically significant values are shown in bold font and underlined.

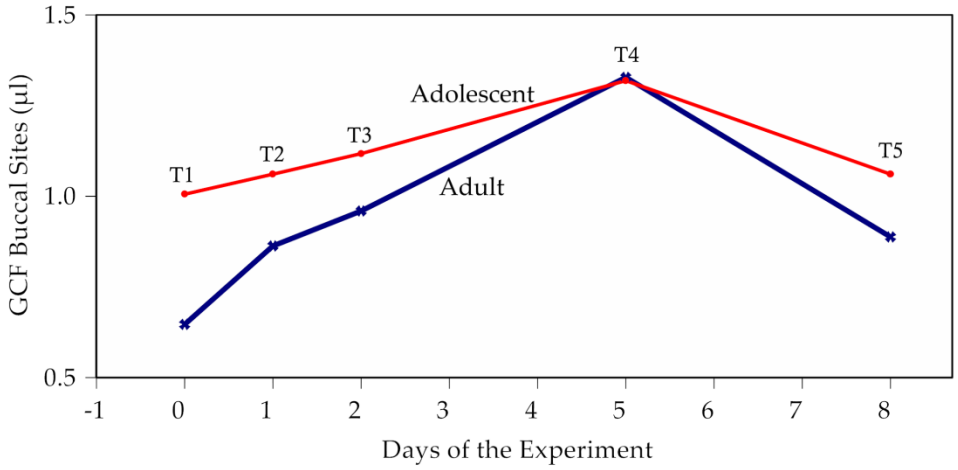

Figure 28. Plot of the volumes of GCF from the buccal sites by age category. The least-squares means are plotted for the five examinations. The MANOVA result was not statistically significant (P = 0.1167), though the LS means tend to be higher in adolescents than adults.

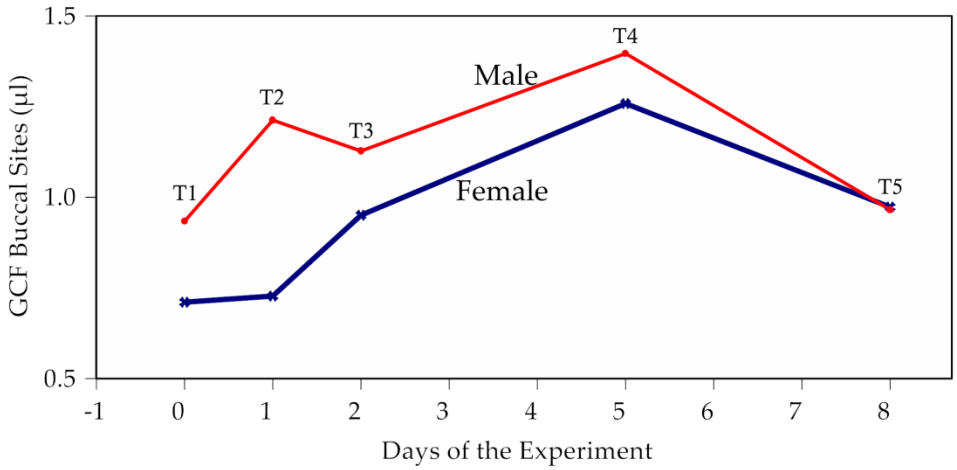

Figure 29. Plot of the volumes of GCF from the buccal sites of the premolars by sex.

The least-squares means are plotted for the five examinations. 'Sex' was not significant across the five tests (P = 0.0686).

Table 21. Results of MANOVA testing for a difference in concentrations of GCF depending on the sex of the subjects.

Source	F-ratio	df	P-value[1]
Between Subjects			
Sex	3.46	1, 51	0.0686
Within Subjects			
Time	4.44	4, 48	**0.0039**
Time-x-Sex	1.24	4, 48	0.3051

[1]Statistically significant values are shown in bold font and underlined.

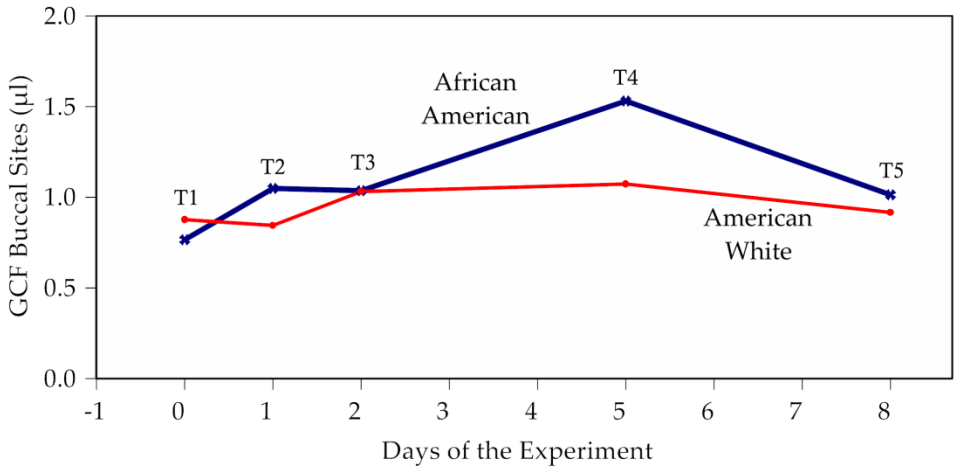

Figure 30. Plot of the volumes of GCF from the buccal sites by race.
The least-squares means are plotted for the five examinations, but do not achieve statistical significance (P = 0.2475).

Table 22. Results of MANOVA testing for a difference in concentrations of GCF at the buccal sites depending on the race of the subjects.

Source	F-ratio	df	P-value[1]
Between Subjects			
Race	1.37	1, 51	0.2475
Within Subjects			
Time	4.45	4, 48	**0.0039**
Time-x-Race	2.00	4, 48	0.1096

[1]Statistically significant values are shown in bold font and underlined.

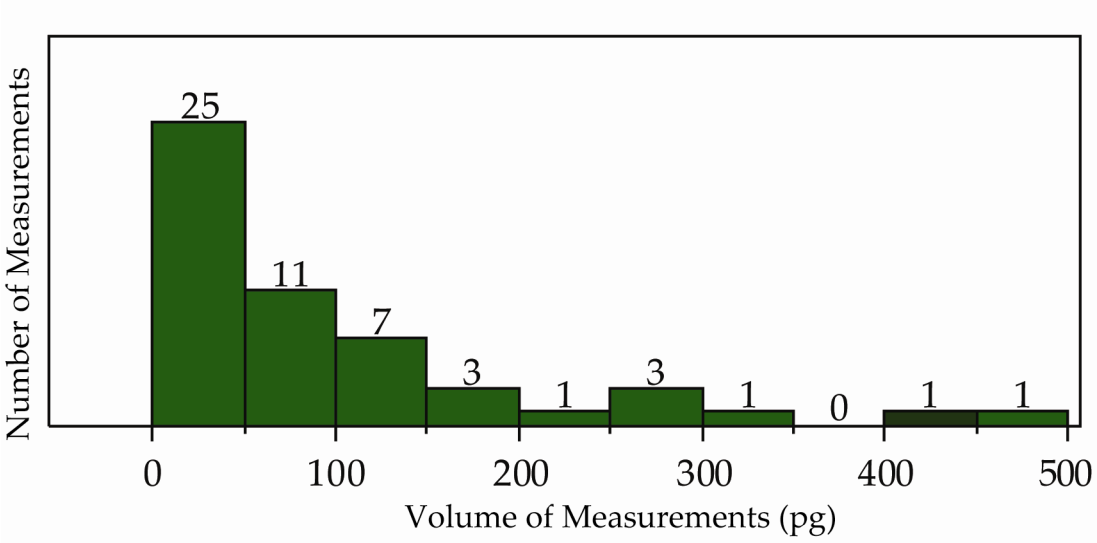

Figure 31. Distribution of RANKL in the combined counts of the five examinations.
The average of the two readings was averaged for each measurement, then T1 through T5 were summed for each person. Clearly, the distribution is positively skewed, so standard parametric statistics that depend on a normal distribution would be suspect.

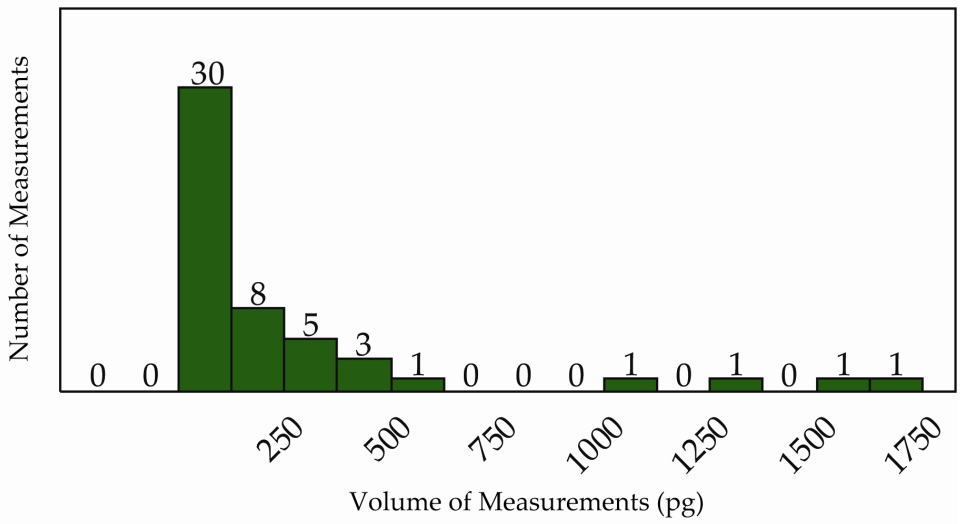

Figure 32. Distribution of OPG in the combined counts of the five examinations.

The point is simply to appreciate the non-normal distribution of the distribution. Clearly, the distribution is positively skewed, so standard parametric statistics that depend on a normal distribution would be suspect.

OPG values were less than 100, but a few exceeded that, rising to a maximum of 345. For OPG, skewness (**Figure 32**) was 6.69, and for kurtosis it was 57.69.

OPG and RANKL: Duplicate Measures

An early point of interest was to test for differences between the repeated samples. There was enough liquid from the dilutions of the gingival crevicular fluid to divide the volume and determine the readings twice. The question was: How duplicative were the two runs? Paired t-tests were calculated, but they are suspect because the data are highly skewed (positively). More reliably, the nonparametric Wilcoxon signed rank test (Castellan and Siegel 1988) was used to test for a systematic difference between readings. **Table 23** lists the results for the OPG concentrations, and **Table 24** does likewise for the RANKL data. The five P-values in each table all were well above alpha (0.05), confirming the absence of any directional effect. Also, all of the product-moment correlations were above 0.90 for the OPG values, but noticeably lower (around 0.80) for the RANKL concentrations. These differences in repeatability probably stem from greater difficulties with the RANKL assay. Since each GCF sample was well homogenized, there was no reason to suspect any difference between first and second readings, but these tests were conducted for completeness.

The two tests for a difference between the duplicated OPG reading (**Table 23**) and RANKL (**Table 24**) readings are shown at the bottom of the tables. A paired t-test was calculated for each of the five examinations, and no test was close to statistical significance for OPG. The same non-significant results were obtained from the five Wilcoxon signed-rank tests.

A comparable absence of significance occurred for the duplicated RANKL readings (**Table 24**). None of the five paired t-tests and none of the Wilcoxon tests approached statistical significance. These negative results hardly come as a surprise, but they do confirm the duplicative nature of the tests. For OPG, the mean differences between readings ranged between 1 and 6. For RANKL, the mean differences between readings range from 1 to 24. Individual readings, of course, differed more widely.

Normality of Distributions

It was of interest whether the observed distributions for OPG and RANKL could be normalized by a transformation. We followed suggestions by Sokal and Rohlf (1995). Looking at the OPG readings first, the readings for all five examinations were combined, and skewness was 6.6931. The formula for the standard error of skewness (Sokal and Rohlf 1995) is

Table 23. Descriptive statistics and result of Wilcoxon's signed rank tests for the similarity of OPG concentrations in the repeated readings at each examination.

Statistic	Exam 1	Exam 2	Exam 3	Exam 4	Exam 5
OPG first sample	55.7514	39.2821	38.0950	30.3187	32.4065
OPG second sample	49.6072	33.4342	38.9033	29.2378	29.6428
Mean difference	6.1443	5.8479	-0.8083	1.0809	2.7637
Std Error	6.4193	3.6897	3.3347	3.9790	3.8969
Upper 95%	19.0316	13.2552	5.8864	9.0690	10.5870
Lower 95%	-6.7430	-1.5595	-7.5031	-6.9073	-5.0596
n	52	52	52	52	52
Correlation	0.955	0.959	0.965	0.904	0.935
Paired t-test	0.957	1.585	-0.242	0.272	0.709
df	51	51	51	51	51
P-Value (two tail)	0.3430	0.1192	0.8094	0.7870	0.4814
Wilcoxon Signed Rank Test Statistic S	-35.50	67.00	-0.50	-89.50	5.50
P-Value (two tail)	0.6933	0.4249	0.9951	0.3178	0.9377

Table 24. Descriptive statistics and result of Wilcoxon's signed rank tests for the similarity of RANKL concentrations in the repeated readings at each examination.

Statistic	Exam 1	Exam 2	Exam 3	Exam 4	Exam 5
RANKL first sample	25.5815	15.5958	14.2113	16.8944	173.3650
RANKL second sample	33.8721	14.6634	13.5366	16.9464	196.8850
Mean difference	-8.2906	0.9323	0.6746	-0.0520	-23.5190
Std Error	11.0092	1.7835	1.6817	1.4373	28.3811
Upper 95%	13.8114	4.5128	4.0507	2.8335	33.4583
Lower 95%	-30.3930	-2.6482	-2.7015	-2.9376	-80.4970
n	52	52	52	52	52
Correlation	0.52759	0.74388	0.89723	0.89982	0.58604
Paired t-test	-0.75306	0.52276	0.401171	-0.03619	-0.82869
df	51	51	51	51	51
P-Value (two tail)	0.4549	0.6034	0.6900	0.9713	0.4111
Wilcoxon Signed Rank Test Statistic S	59.50	47.50	58.00	11.00	-128.00
P-Value (two tail)	0.4635	0.4982	0.4254	0.8996	0.1233

$$\sqrt{\frac{6n(n-1)}{(n-2)(n+1)(n+3)}} \approx \sqrt{\frac{6}{n}}$$

where n is the sample size, so the critical test for the raw data was exceedingly large (t = 44.1). With the base 10-logarithmic transformation, skewness became 0.8530, and the critical test was 5.61. Recall that the logarithm of zero is undefined—and several values were "zero" because they were below the level of detectability of the assay—so a small integer (5) was added to all values prior to the transformation so all values were above zero and the logarithm could be taken. There was a major reduction in skewness with the log-transformation, but it was still highly significant statistically. The log-log transformation produced skewness of 0.1137, with a corresponding critical test of t = 0.75, which was not a significant departure from normality.

For the RANKL distribution, skewness for the raw scores was 37.1680. The log-transform created a skewness of 0.5914, and the corresponding critical ratio was t = 1.95. This ratio was fractionally less than the critical value of t (= 1.96). In turn, the log-log transform made skewness 0.0327, so the critical ratio was t = 0.11, which was well below the level of significance (alpha = 0.05).

Consequently, use of the log-log transformation (base 10) adequately normalized the distributions regarding skewness and kurtosis, making it reasonable to pursue parametric statistical analysis, both for OPG and RANKL. This creates minor problems in that the data have to be transformed back to nanograms (and then subtracting the offset of 5), but it permits the legitimate use of parametric statistics.

OPG and RANKL Concentrations across Time

The nature of the study, simplified, was (1) to measure the pretreatment, baseline level, then (2) apply tension to the crowns of four teeth for a period of time (T2 through T4), and finally (3) test the post-treatment concentrations three days after removal of the tensioned spring (examination T5). See **Figure 33**. The data are serial (repeated measures), so the statistical method needs to match this.

Multivariate analysis of variance (MANOVA) was used to look at the change in least-squares means over time (of the log-log transformed data). As shown in **Figure 34**, the least-squares means for RANKL were highest at baseline, and then dropped to a low at T2, slowly rising thereafter, but returning fully to baseline by T5. Statistically, though, there was so much inter-individual variation that these changes across time are only marginally significant statistically (F = 2.77; df = 4 and 48; P = 0.0377). The concentrations decreased from T1 to a low at T3, then increased to essentially baseline by T5.

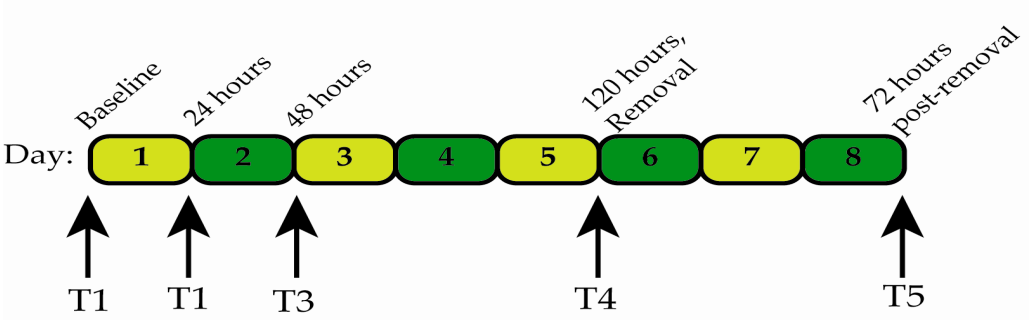

Figure 33. Schematic showing the time-line of the experiment.
Each box is 1 day, with a total duration of 8 days and five examinations (T1 through T5).

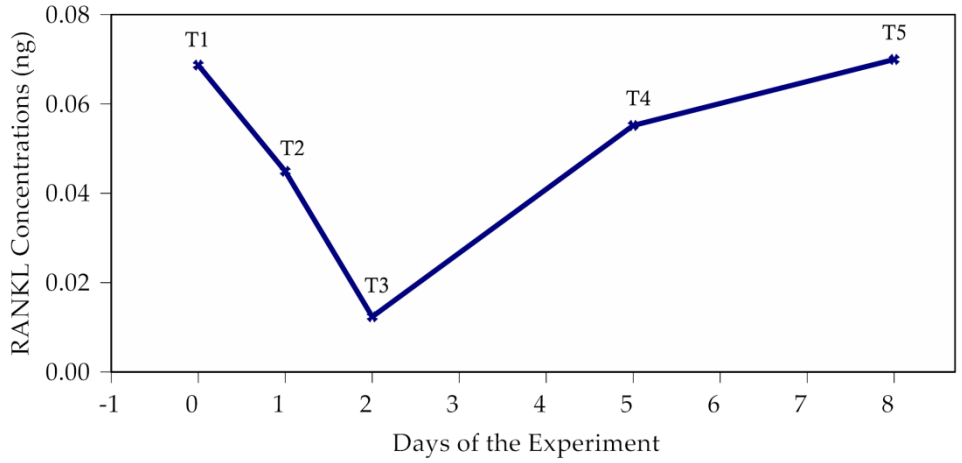

Figure 34. Plot of the least-squares means by examination for the concentrations of RANKL in the total sample (with log-log transformation).
The differences, in this parametric model did achieve statistical significance by MANOVA (P = 0.0377). The "LS means" are plotted, which are the least-squares means provided by the MANOVA output.

The matrix of P-values from paired t-tests (**Table 25**) showed that the RANKL concentration by T3 was significantly lower than baseline (T1 and T2). Concentrations by examinations T4 and T5 were statistically non-significant compared to T1. In other words, the change in RANKL concentration was due to the lower concentration at T3. These findings were unanticipated because the increase in osteoclastogenesis induced by a transpalatal spring should increase the concentrations of RANKL—RANKL should increase while OPG should decrease, or RANKL could remain constant and OPG concentrations decrease, which would also lead to a higher RANKL/OPG ratio thereby promoting bone resorption (*e.g.*, Meikle 2007).

The descriptive statistics for the log-transformed RANKL concentrations are provided in **Table 26**.

The temporal changes in OPG (as assessed parametrically) are graphed in **Figure 35**. The highest average concentration was at pretreatment. After tension was placed on the teeth, the average concentration decreased and did not return to the baseline level by T5. Statistically, the MANOVA result was significant statistically for these OPG concentrations ($F = 3.63$; $df = 4$ and 48; $P = 0.0115$). This trend is as expected because OPG should decrease in response to the orthodontic force from the transpalatal spring.

To evaluate the source of significance among the five examinations, a matrix of paired t-tests was calculated (**Table 27**). These values are two-tail P-values. In this instance, all of the significant differences involved examination T1; that is, there was a significant drop in OPG concentration when the transpalatal spring was applied, and the four subsequent values remained significantly lower than the T1 value thereafter (T2 through T5). The T5 concentration was the lowest of the five, so there was no suggestion that OPG was returning to its baseline value by the end of the experiment.

The descriptive statistics for OPG concentrations are listed in **Table 28**.

OPG and RANKL: Sources of Variation

A major interest in this study was to test for (1) sex, (2) age, and/or (3) race differences in the levels of RANK and/or OPG.

Concentrations by Sex

We begin with the examination of the subject's sex. Does the sex of the person affect the concentration of RANKL and/or OPG across the five examinations? The log-log transformations of the RANKL concentrations were analyzed. Results of the MANOVA test for RANKL are shown in **Table 27**. The differences in the concentrations

Table 25. Matrix of P-values to interpret the source of significance of the MANOVA test for differences in the concentrations of RANKL.

	Group[1]			
Group	T1	T2	T3	T4
T2	0.3464			
T3	**0.0143**	0.0873		
T4	0.4970	0.6207	**0.0040**	
T5	0.9499	0.2434	**0.0113**	0.4592

[1]Statistically significant values are shown in bold font and underlined.

Table 26. Descriptive statistics for the log-transformed concentrations of RANKL at the five examinations.

Statistic	T1	T2	T3	T4	T5
Mean	29.727	0.045	0.012	0.055	0.070
Std Deviation	58.142	0.142	0.137	0.133	0.148
Std Error of Mean	8.063	0.020	0.019	0.018	0.021
Upper 95% Mean	45.914	0.084	0.050	0.092	0.111
Lower 95% Mean	13.540	0.005	-0.026	0.018	0.029
n	52	52	52	52	52

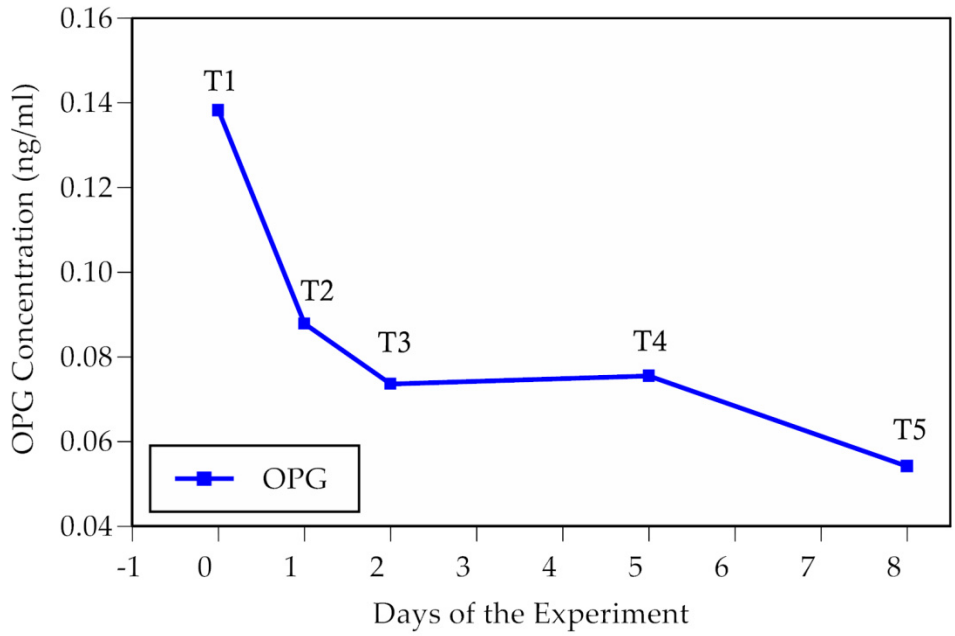

Figure 35. Plot of the least-squares means by examination for the concentrations of OPG in the total sample (with log-log transformation). The differences in this parametric model were significant by MANOVA (P = 0.0115). Parenthetically, without transforming the data, this result was not significant (P = 0.1156).

Table 27. Matrix of P-values to interpret the source of significance of the MANOVA test for differences in the concentrations of OPG.

	Group[1]			
Group	T1	T2	T3	T4
T2	**0.0478**			
T3	**0.0142**	0.2689		
T4	**0.0170**	0.5316	0.9113	
T5	**0.0005**	0.0693	0.3153	0.2805

[1]Statistically significant differences are printed in bold and underlined.

Table 28. Descriptive statistics for the log-transformed concentrations of OPG at the five examinations.

Statistic	T1	T2	T3	T4	T5
Mean	0.138	0.088	0.074	0.076	0.054
Std Deviation	0.192	0.164	0.164	0.149	0.166
Std Error of Mean	0.027	0.023	0.023	0.021	0.023
Upper 95% Mean	0.192	0.134	0.119	0.117	0.101
Lower 95% Mean	0.085	0.042	0.028	0.034	0.008
n	52	52	52	52	52

of RANKL were interpreted using the matrix of P-values listed in **Table 29**. Notice that the inter-examination differences were driven more by the males in the sample, while the female subset did not contribute significantly. This test shows that 'sex' does not affect the concentration of RANKL as examined here (P = 0.1513), though the concentrations were consistently a bit lower in females than males (**Figure 36**). Change in OPG across time, of course, also was significant as discussed earlier (P = 0.0394).

The arithmetic means of these variables (total RANKL, by sex) are listed in **Table 30**.

Looking at the same test for OPG, the results (**Table 31**) disclosed a marginally-significant difference in OPG concentrations by sex (P = 0.0477). As shown in **Figure 37**, males consistently had the higher concentration at all five examinations. Again, the difference across examinations (time) was significant statistically (P = 0.0138). In the sample for girls the matrix of P-values (**Table 32**) shows that the source of significance primarily involves the high levels of RANKL at T5. In contrast, the data for males (**Table 33**) indicates that their difference in OPG primarily was the high, pretreatment level at T1.

Concentrations by Age

The second variable to be examined was age, where we have dichotomized into adolescents (under 18 years) and adults (over 18 years). However, it is worthwhile first to look at the range of ages expressed in years. The descriptive statistics for the concentrations of OPG by age grade are provided in **Table 34**. These values are, of course, log-transformed. Concentrations (the dependent variable) were regressed on ages of the subjects (the independent variable). The associations at each of the five examinations are graphed in **Figures 38 through 42**.

Table 35 shows the MANOVA results of testing for RANKL concentrations by

Table 29. MANOVA results testing for a sex difference in the concentrations of RANKL over the five examinations.

Source	F-ratio	df	P-value
Between Subjects			
Sex	2.12	1, 50	0.1513
Within Subjects			
Time	2.74	4, 47	**0.0394**
Time-x-Sex	0.26	4, 47	0.9044

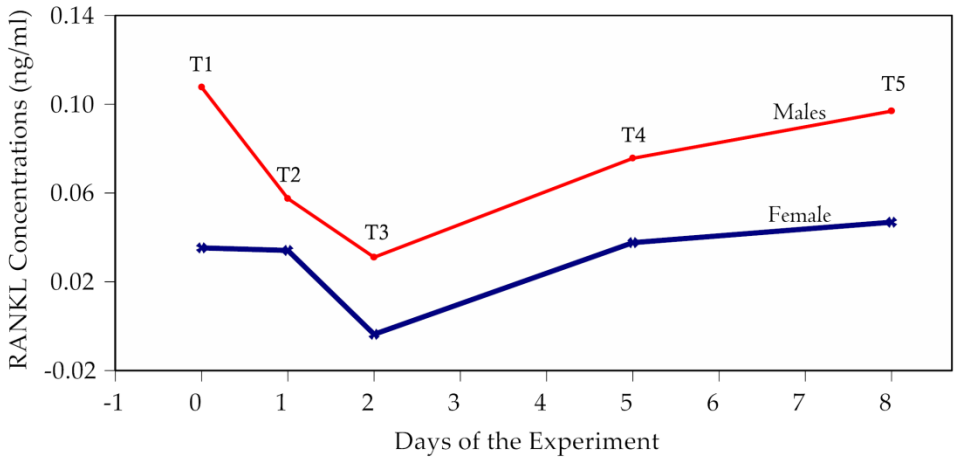

Figure 36. Plot of a MANOVA for the concentrations of RANKL across examinations by sex.
Plot is of the log-log transformation of RANKL (plus 5). Males consistently exhibited higher mean concentrations across all five examinations, though the difference between sexes was not significant (P = 0.1513).

Table 30. Arithmetic means for RANKL, by sex, at the five examinations.

Statistic	Female	Male
	Total GCF d1	
Mean	1.740	2.006
Std Deviation	1.027	0.884
Std Error of Mean	0.182	0.173
Upper 95% Mean	2.111	2.363
Lower 95% Mean	1.370	1.649
n	32	26
	Total GCF d2	
Mean	2.544	2.939
Std Deviation	1.010	1.185
Std Error of Mean	0.191	0.237
Upper 95% Mean	2.936	3.428
Lower 95% Mean	2.153	2.450
n	28	25
	Total GCF d3	
Mean	3.008	2.914
Std Deviation	1.307	1.067
Std Error of Mean	0.247	0.213
Upper 95% Mean	3.514	3.355
Lower 95% Mean	2.501	2.474
n	28	25
	Total GCF d4	
Mean	3.247	3.271
Std Deviation	1.339	1.450
Std Error of Mean	0.253	0.290
Upper 95% Mean	3.766	3.870
Lower 95% Mean	2.728	2.673
n	28	25
	Total GCF d5	
Mean	2.383	2.224
Std Deviation	1.277	1.189
Std Error of Mean	0.241	0.238
Upper 95% Mean	2.879	2.715
Lower 95% Mean	1.888	1.733
n	28	25

The units are ng/ml. These are identical to the values plotted in **Figure 36**.

Table 31. MANOVA results testing for a sex difference in the concentrations of OPG over the five examinations.

Source	F-ratio	df	P-value[1]
Between Subjects			
Sex	4.1221	50	**0.0477**
Within Subjects			
Time	3.5075	4, 47	**0.0138**
Time-x-Sex	1.2452	4, 47	0.3049

[1]Statistically significant values are shown in bold font and underlined.

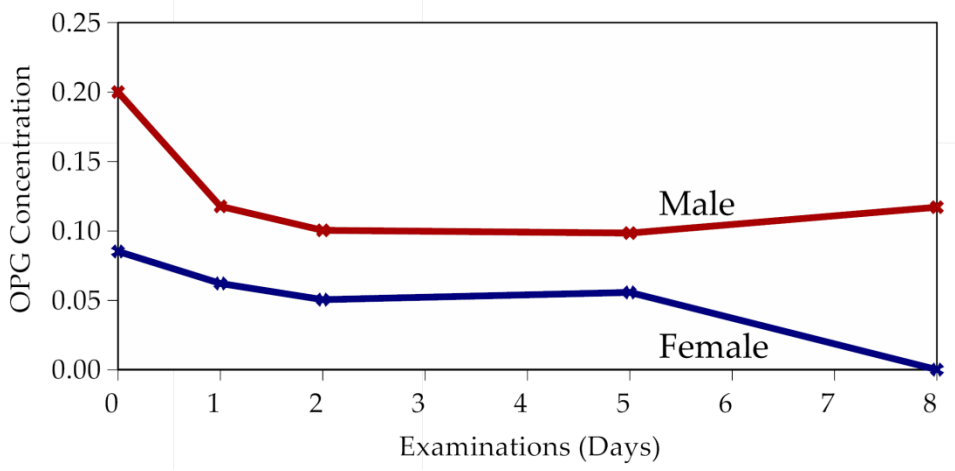

Figure 37. Plot of a MANOVA for the concentrations of OPG across examinations by sex.
Plot is of the log-log transformation of OPG (plus 5). Males consistently exhibited higher concentrations across all five examinations.

Table 32. Matrix of P-values to interpret the source of significance of the MANOVA test for differences in the concentrations of RANKL.

	Group				
Group[1]	T1	T2	T3	T4	T5
T1	--	0.9740	0.2547	0.9312	0.6798
T2	0.1749	---	0.1941	0.9125	0.6553
T3	**0.0128**	0.2792	---	0.0502	0.1289
T4	0.2785	0.4869	**0.0380**	---	0.7499
T5	0.7146	0.2381	**0.0360**	0.4492	---

[1]Results for girls are shown in the upper right half of the matrix; boys are listed in the lower left diagonal.

Table 33. Matrix of P-values to interpret the source of significance of the MANOVA test for differences in the concentrations of OPG.

	Group[1]				
Group	T1	T2	T3	T4	T5
T1	---	0.5629	0.3762	0.4346	**0.0272**
T2	**0.0067**	---	0.5151	0.8251	**0.0256**
T3	**0.0041**	0.3667	---	0.8356	0.0507
T4	**0.0053**	0.4747	0.9334	---	**0.0444**
T5	**0.0035**	0.9794	0.5689	0.5061	---

[1]Results for girls are shown in the upper right half of the matrix; boys are listed in the lower left diagonal.

Table 34. Descriptive statistics, by sex, for the levels of total OPG at each examination.

Statistic	Female	Male
	loglog(OPG) T1	
Mean	0.085	0.200
Std Deviation	0.201	0.165
Std Error of Mean	0.038	0.034
Upper 95% Mean	0.163	0.269
Lower 95% Mean	0.007	0.131
n	28	24
	loglog(OPG) T2	
Mean	0.062	0.118
Std Deviation	0.156	0.172
Std Error of Mean	0.029	0.035
Upper 95% Mean	0.123	0.191
Lower 95% Mean	0.002	0.045
n	28	24
	loglog(OPG) T3	
Mean	0.051	0.100
Std Deviation	0.154	0.176
Std Error of Mean	0.029	0.036
Upper 95% Mean	0.110	0.175
Lower 95% Mean	-0.009	0.026
n	28	24
	loglog(OPG) T4	
Mean	0.056	0.099
Std Deviation	0.147	0.152
Std Error of Mean	0.028	0.031
Upper 95% Mean	0.113	0.163
Lower 95% Mean	-0.001	0.034
n	28	24
	loglog(OPG) T5	
Mean	0.000	0.117
Std Deviation	0.150	0.165
Std Error of Mean	0.028	0.034
Upper 95% Mean	0.059	0.187
Lower 95% Mean	-0.058	0.047
n	28	24

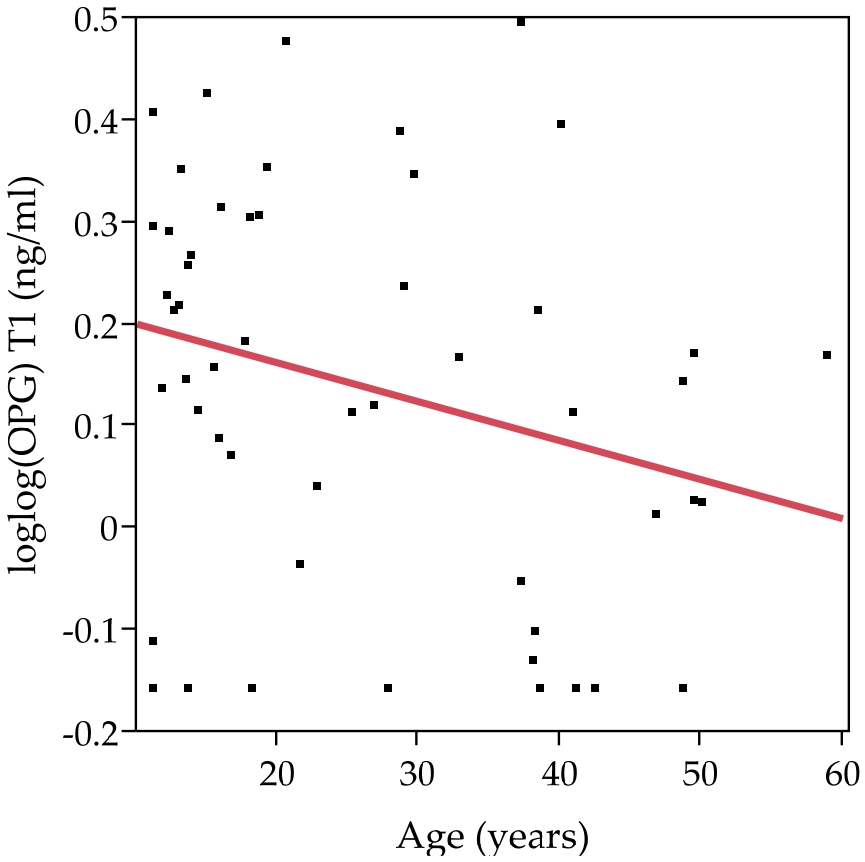

Figure 38. The plot of OPG at T1 regressed on the subject's age in years. The regression equation is loglog(OPG) = 0.2389 - 0.0038(age), where the intercept ($P < 0.0001$) and the regression coefficient ($P = 0.0499$) are statistically significant.

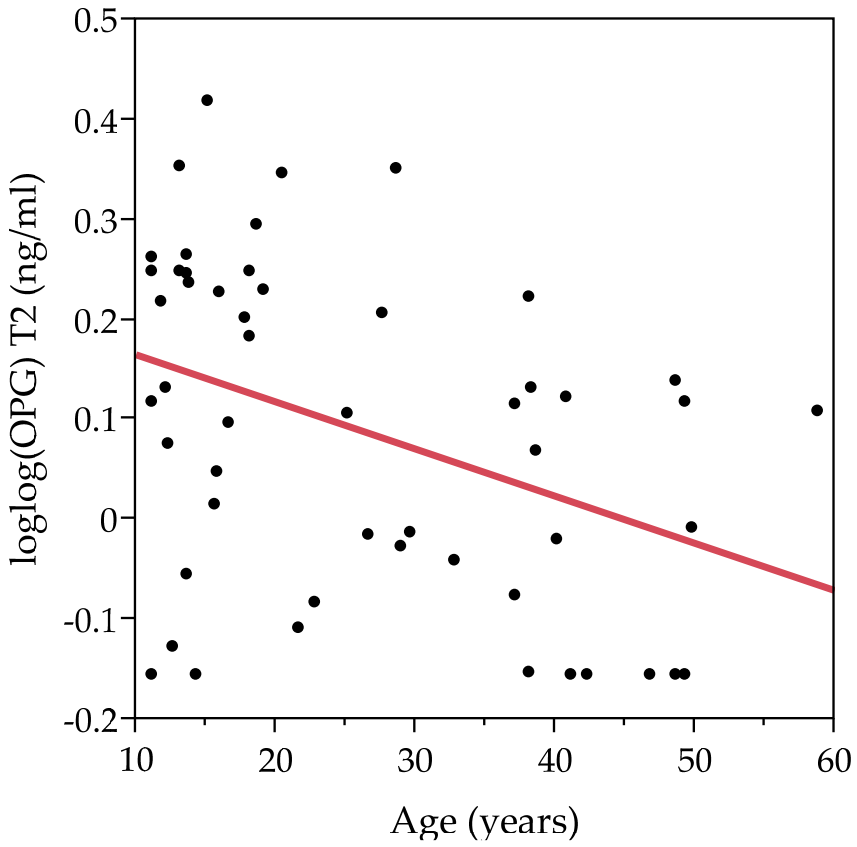

Figure 39. The plot of OPG at T2 regressed on the subject's age in years.
The regression equation is loglog(OPG) = 0.2120 - 0.0047(age), where the intercept ($P < 0.0001$) and the regression coefficient ($P = 0.0038$) are statistically significant.

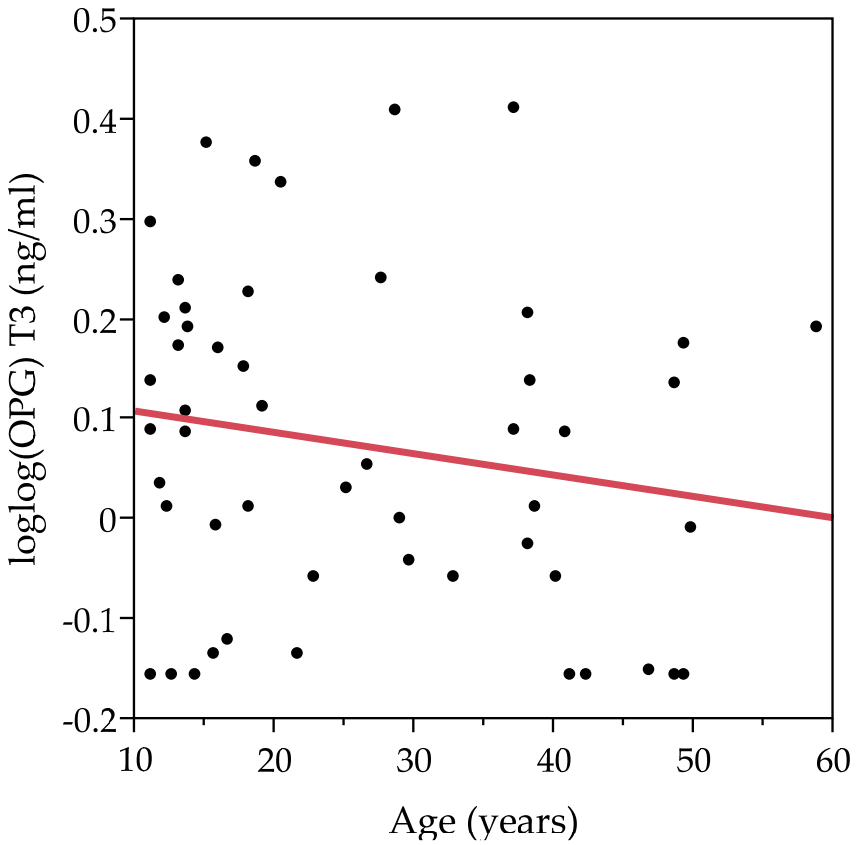

Figure 40. The plot of OPG at T3 regressed on the subject's age in years.
The regression equation is loglog(OPG) = 0.1298 - 0.0021 (age), where the intercept (P = 0.0113) is statistically significant but the regression coefficient (P = 0.2057) is not.

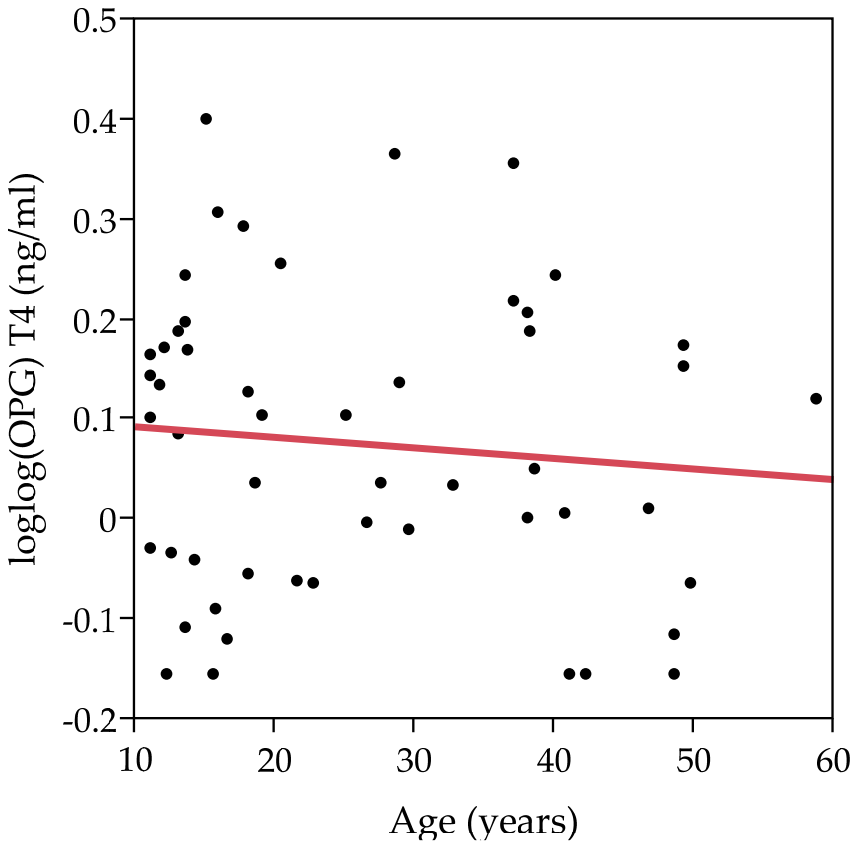

Figure 41. The plot of OPG at T4 regressed on the subject's age in years.
The regression equation is loglog(OPG) = 0.1034 - 0.0011(OPG), where the intercept (P = 0.0270) is statistically significant but the regression coefficient (P = 0.4927) is not.

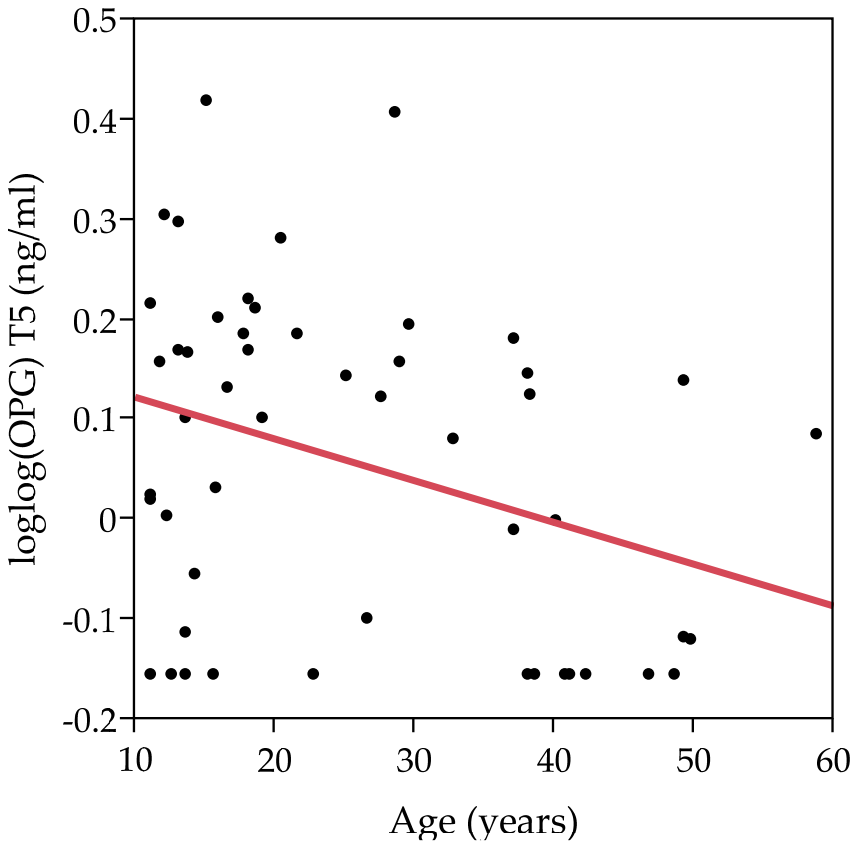

Figure 42. The plot of OPG at T5 regressed on the subject's age in years.
The regression equation is loglog(OPG) = 0.1641 - 0.0042, where the intercept (P = 0.0112) is statistically significant and so is the regression coefficient (P = 0.0124).

Table 35. Results of MANOVA testing for differences in concentrations of RANKL depending on the subject's age category.

Source	F-ratio	df	P-value
Between Subjects			
Age Category	0.0034	1, 50	0.9535
Within Subjects			
Time	2.702	4, 47	**0.0416**
Time-x-Age Category	0.4634	4, 47	0.7622

age grade. There was no evidence of a difference between age grades (P = 0.9535), and this is shown graphically in **Figure 43**. Indeed, the means were very close together at T1, T3, and T5, and the concentrations by age grade were in opposite rankings at T2 and T4.

The descriptive statistics for the concentrations of RANKL by age grade are provided in **Table 36**. These values are, of course, log-transformed.

Turning to the RANKL data, the regression of age of the subject at examination is regressed on the RANKL concentration as it was for OPG, and these results are shown in **Figures 44 through 48.**

The results of linear regression analyses for OPG are listed in **Table 37**, and the interesting finding is that three of the five tests (T1, T2, and T5) achieved statistical significance In each case, as shown in the graphs (**Figures 38 through 42**), the association was negative: Older subjects (adults) had lower concentrations of OPG than the adolescents. (The intercepts also are all significant, but they have no relevance in these tests.) Visually assessing the relationships between OPG and age shows no inflection point; the decrease appears to be linear with age, though there is a good deal of inter-individual variation.

The use of the two age categories (adolescent or adult) in place of the continuous variable (age in years) was tested. Results of ANOVA for each of the five examinations

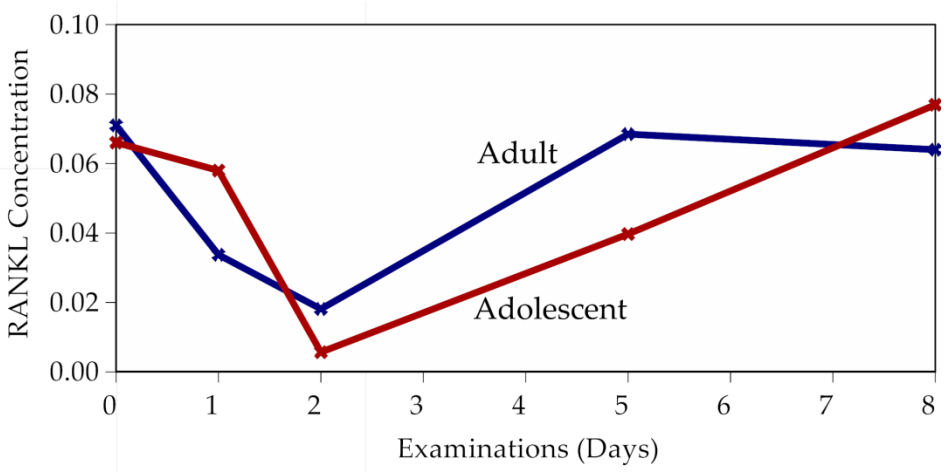

Figure 43. Plot LS means for the concentrations of RANKL in the two age grades across the five examinations.
Values are the log-log transformation of the raw values (plus 5). There seems to be no patterned difference by age grade across time.

Table 36. Descriptive statistics, by age grade, for the levels of RANKL at each examination.

Statistic	Adult	Child
	log(RANKL) T1	
Mean	0.071	0.066
Std Deviation	0.164	0.152
Std Error of Mean	0.031	0.031
Upper 95% Mean	0.135	0.130
Lower 95% Mean	0.007	0.002
n	28	24
	log(RANKL) T2	
Mean	0.034	0.058
Std Deviation	0.148	0.136
Std Error of Mean	0.028	0.028
Upper 95% Mean	0.091	0.115
Lower 95% Mean	-0.024	0.001
n	28	24
	log(RANKL) T3	
Mean	0.018	0.006
Std Deviation	0.141	0.135
Std Error of Mean	0.027	0.028
Upper 95% Mean	0.073	0.063
Lower 95% Mean	-0.036	-0.051
n	28	24
	log(RANKL) T4	
Mean	0.068	0.040
Std Deviation	0.130	0.138
Std Error of Mean	0.025	0.028
Upper 95% Mean	0.119	0.098
Lower 95% Mean	0.018	-0.019
n	28	24
	log(RANKL) T5	
Mean	0.064	0.077
Std Deviation	0.145	0.155
Std Error of Mean	0.027	0.032
Upper 95% Mean	0.120	0.142
Lower 95% Mean	0.008	0.012
n	28	24

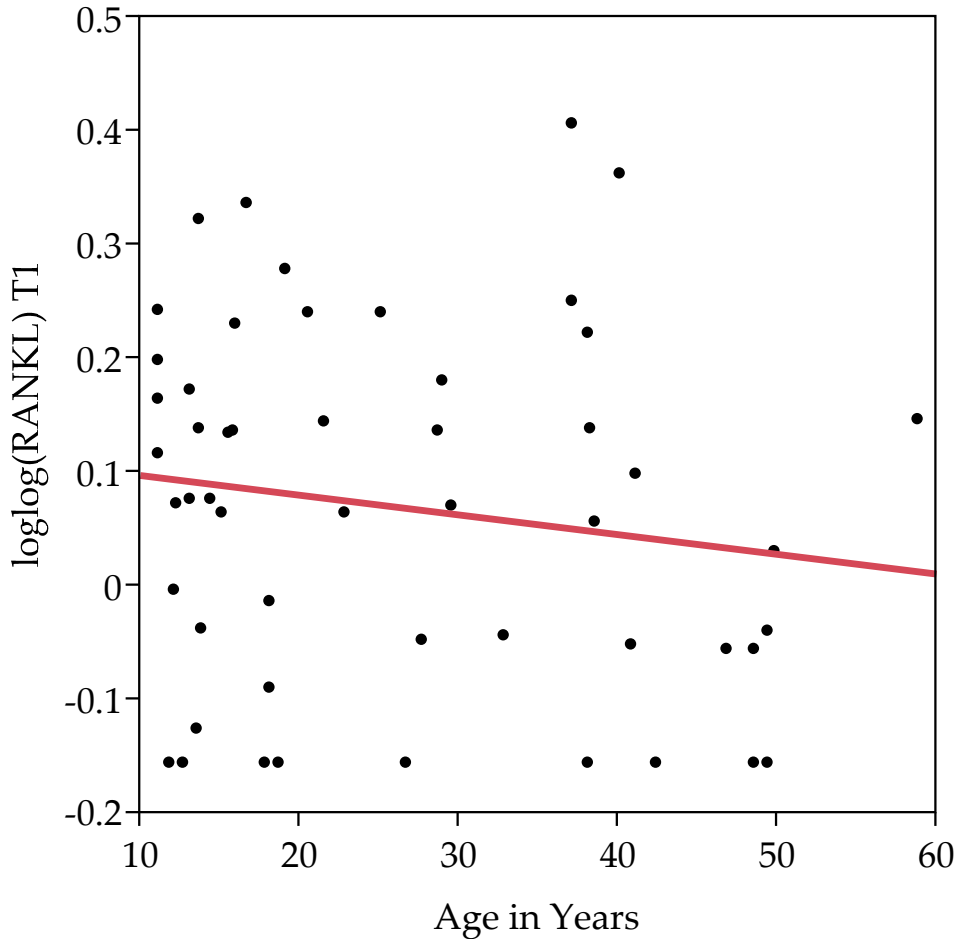

Figure 44. The plot of RANKL at T1 regressed on the subject's age in years. The regression equation is loglog(RANKL) = 0.114 - 0.0017, where the intercept (P = 0.0196) but not the regression coefficient (P = 0.2840) is statistically significant.

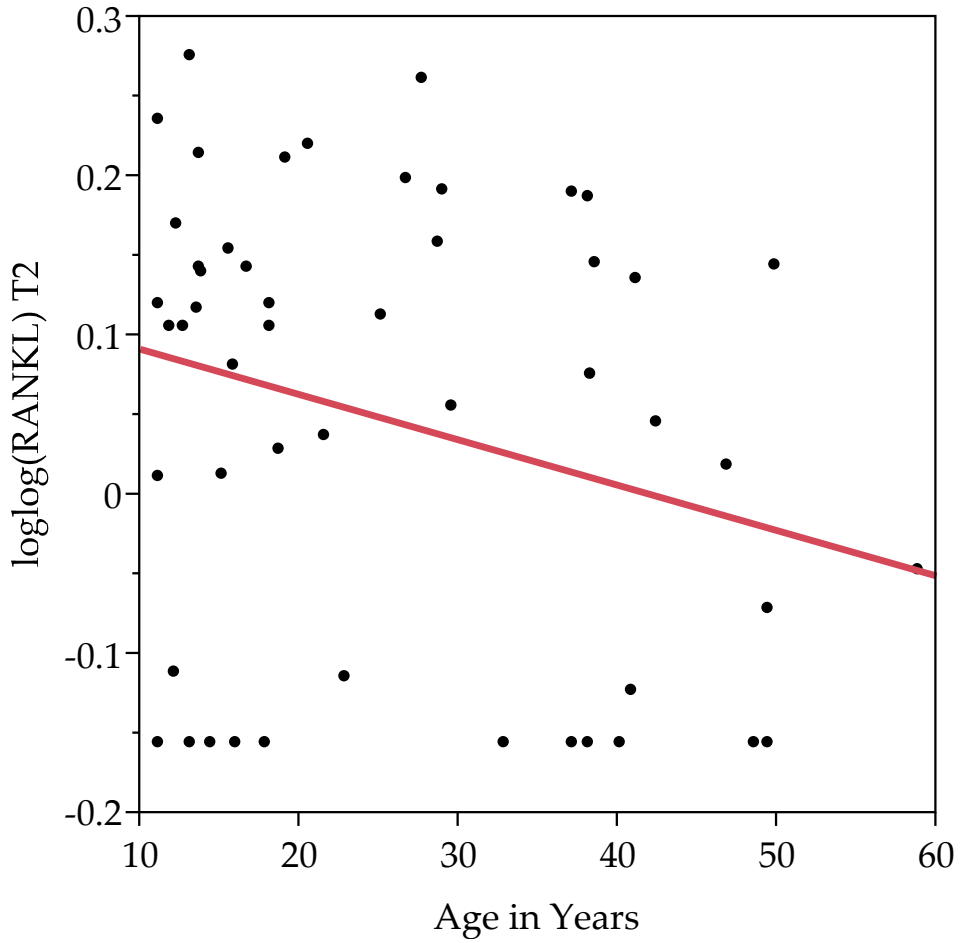

Figure 45. The plot of RANKL at T2 regressed on the subject's age in years. The regression equation is loglog(RANKL) = 0.1199 - 0.0028, where the intercept (P = 0.0058) and the regression coefficient (P = 0.0478) are statistically significant.

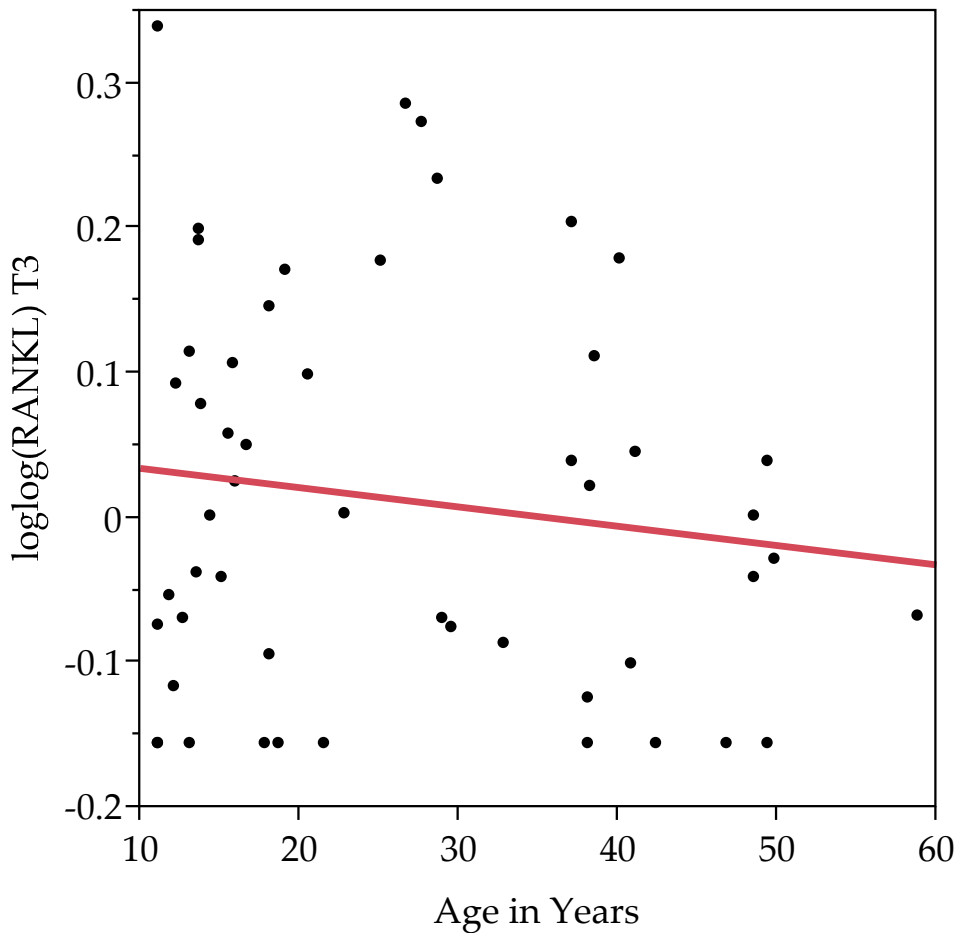

Figure 46. The plot of RANKL at T3 regressed on the subject's age in years. The regression equation is loglog(RANKL) = 0.0474 - 0.0013, where neither the intercept (P = 0.2565) nor the regression coefficient (P = 0.3440) is statistically significant.

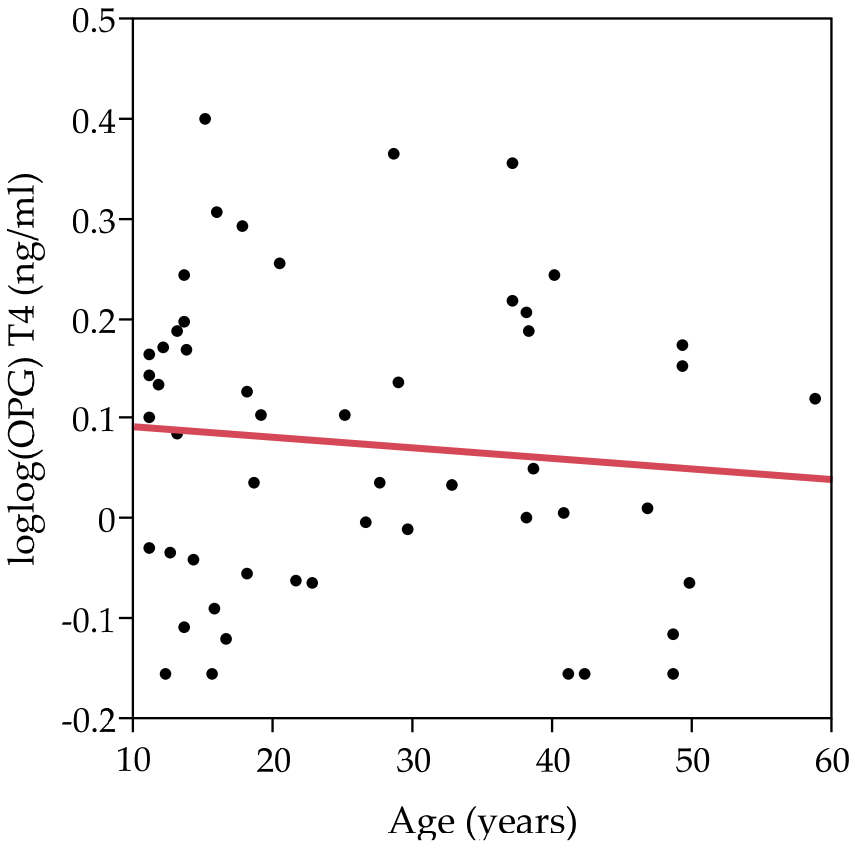

Figure 47. The plot of RANKL at T4 regressed on the subject's age in years. The regression equation is loglog(RANKL) = 0.0789 - 0.0009, where neither the intercept (P = 0.0563) nor the regression coefficient (P = 0.5111) is statistically significant.

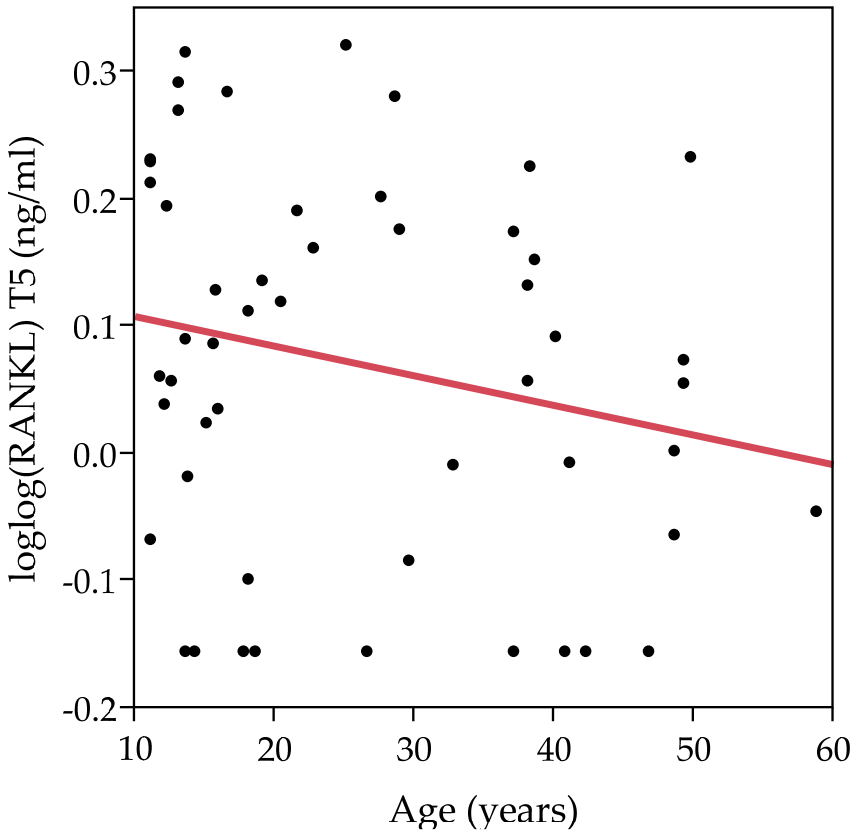

Figure 48. The plot of RANKL at T5 regressed on the subject's age in years. The regression equation is loglog(RANKL) = 0.1312 - 0.0023, where the intercept (P = 0.0044) but not the regression coefficient (P = 0.1236) is statistically significant.

Table 37. Results of linear regression at each of the five examinations testing for an association between subject's age and concentration of OPG.

Term	Estimate	Std Error	t ratio	P-value[1]
loglog(OPG) T1 by age (years)				
Intercept	0.238927	0.056394	4.24	< 0.0001
Age (years)	-0.003830	0.001904	-2.01	**0.0499**
loglog(OPG) T2 by age (years)				
Intercept	0.211993	0.046088	4.60	<.0001
Age (years)	-0.004720	0.001556	-3.03	**0.0038**
loglog(OPG) T3 by age (years)				
Intercept	0.129828	0.049357	2.63	0.0113
Age (years)	-0.002140	0.001666	-1.28	0.2057
loglog(OPG) T4 by age (years)				
Intercept	0.103365	0.045363	2.28	0.0270
Age (years)	-0.001060	0.001531	-0.69	0.4927
loglog(OPG) T5 by age (years)				
Intercept	0.164051	0.047651	3.44	0.0012
Age (years)	-0.004180	0.001609	-2.60	**0.0124**

[1]The Y-intercepts, though statistically significant, are not highlighted because they have no relevance here, but 3 of the 5 regression coefficients achieved significance (two-tail tests).

is shown in **Table 38**, and results were that just one of the five age categories was statistically significant (T2; P = 0.0084). This means there was less detection capability using the categorical data for age.

Table 39 shows the results of a MANOVA testing for a difference by subject's age (graphed in **Figure 49**). Age is statistically significant (P = 0.0227). **Figure 50** shows that children have higher levels of OPG while the levels in adults are lower.

The descriptive statistics associated with these tests (*i.e.*, OPG against age grade) are listed in **Table 40**.

Black-White Racial Variation

This appears to be the first time for RANKL or OPG to be tested for a race difference. American blacks and whites were compared primarily because of the ready available of these two groups at our orthodontic clinic. MANOVA results for the concentrations of OPG across the five examinations are listed in **Table 41** and graphed in **Figure 51**. There is no suggestion of a race difference (P = 0.8351). The plots of the LS means are virtually superimposed by race.

The descriptive statistics for RANKL concentrations at the five examinations, partitioned by race, are listed in **Table 42**.

Turning to the data for OPG, there also was no statistically significant difference between the races in this analysis (**Table 43**), but the results in **Figure 52** are interesting because the OPG concentrations in blacks all are higher than in whites. There is the possibility, then, that a larger and more homogeneous group (*e.g.*, restricted ages) would produce significant results between these two races.

The descriptive statistics for OPG partitioned by race are listed in **Table 44**.

The RANKL by OPG Ratio

Osteoclastogenesis is characterized by an increase in the concentration of RANKL and a decrease in OPG (Kanzaki *et al.* 2001; Liebbrandt and Penninger 2008), so the ratio of RANKL-to-OPG should increase with progressive osteoclastic activity. **Figure 53** plots the concentrations of RANKL and OPG (in the loglog transformed domain). Broadly, the concentration of OPG decreased with the duration of the force of the transpalatal spring, though there was no apparent recovery after spring removal. RANKL concentration initially dropped but then rose with duration, though it too exhibited little recovery after spring removal. There is the suggestion that these

Table 38. ANOVA results testing for a difference in OPG concentrations between age categories.

Source	df	Mean Square	F Ratio	P-value
One-way analysis of loglog(OPG) T1 by age category				
Age Category	1	0.0654	1.80	0.1861
Error	50	0.0364		
Corrected Total	51			
One-way analysis of loglog(OPG) T2 by age category				
Age Category	1	0.1805	7.53	**0.0084**
Error	50	0.0240		
Corrected Total	51			
One-way analysis of loglog(OPG) T3 by age category				
Age Category	1	0.0266	0.98	0.3267
Error	50	0.0271		
Corrected Total	51			
One-way analysis of loglog(OPG) T4 by age category				
Age Category	1	0.0018	0.08	0.7811
Error	50	0.0228		
Corrected Total	51			
One-way analysis of loglog(OPG) T5 by age category				
Age Category	1	0.0663	2.46	0.1230
Error	50	0.0269		
Corrected Total	51			

Table 39. Results of MANOVA testing for differences in concentrations of OPG depending on the subject's chronological age.

Test	F-ratio	df	P-value
Between Subjects			
Age Category	5.532	1, 50	**0.0227**
Within Subjects			
Time	3.5831	4, 47	**0.0125**
Time-x-Age	2.9508	4, 47	**0.0295**

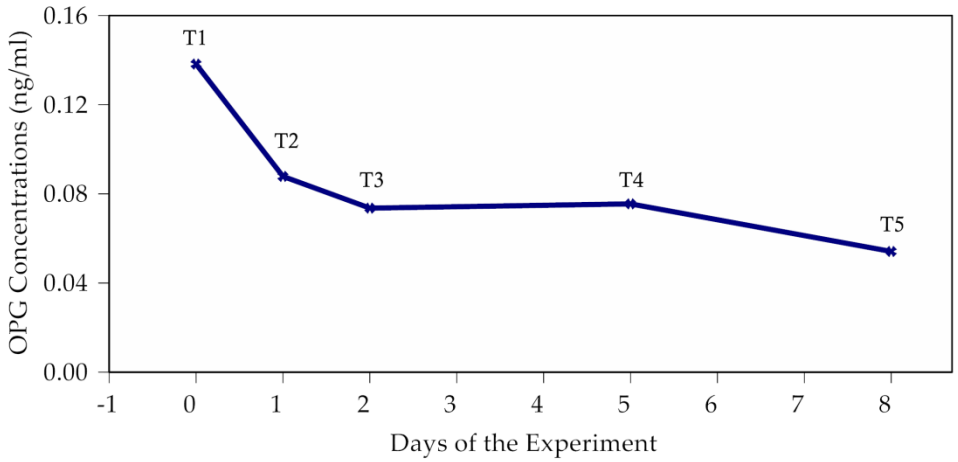

Figure 49. Plot of the least-squares means in the concentrations of OPG pooling the entire sample.
Values are the log-log transformation of OPG (plus 5).

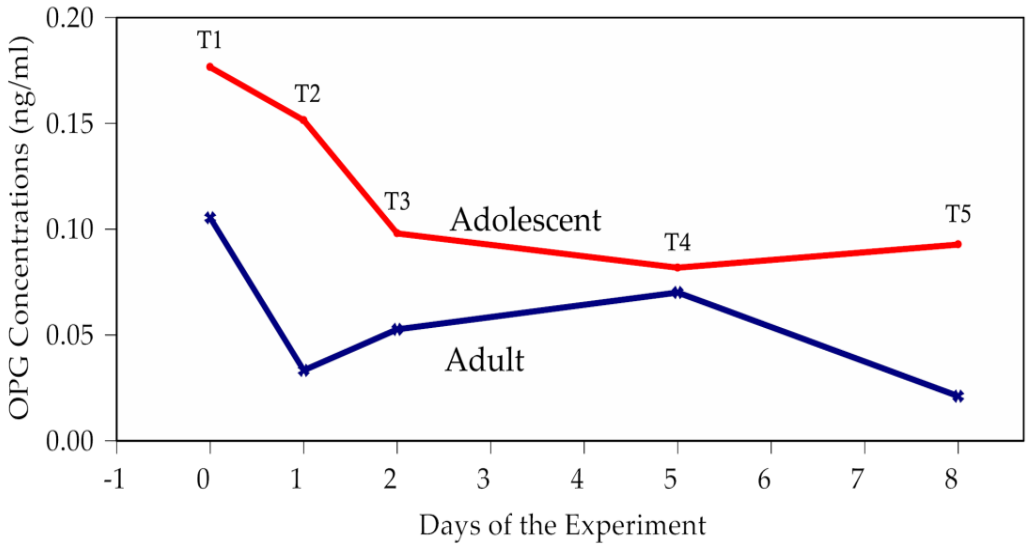

Figure 50. Plot of LS mean concentrations for OPG categorized by subject's age (adolescent, adult).
The concentration in the adolescents is higher at all examinations.

Table 40. Descriptive statistics, by age grade, for the levels of OPG at each examination.

Statistic	Adult	Child
	log(OPG) T1	
Mean	0.105	0.177
Std Deviation	0.205	0.173
Std Error of Mean	0.039	0.035
Upper 95% Mean	0.185	0.250
Lower 95% Mean	0.026	0.103
n	28	24
	log(OPG) T2	
Mean	0.033	0.151
Std Deviation	0.153	0.157
Std Error of Mean	0.029	0.032
Upper 95% Mean	0.093	0.218
Lower 95% Mean	-0.026	0.085
n	28	24
	log(OPG) T3	
Mean	0.053	0.098
Std Deviation	0.168	0.161
Std Error of Mean	0.032	0.033
Upper 95% Mean	0.118	0.166
Lower 95% Mean	-0.012	0.030
n	28	24
	log(OPG) T4	
Mean	0.070	0.082
Std Deviation	0.147	0.156
Std Error of Mean	0.028	0.032
Upper 95% Mean	0.127	0.148
Lower 95% Mean	0.013	0.016
n	28	24
	log(OPG) T5	
Mean	0.021	0.093
Std Deviation	0.165	0.163
Std Error of Mean	0.031	0.033
Upper 95% Mean	0.085	0.162
Lower 95% Mean	-0.043	0.024
n	28	24

Table 41. Results of MANOVA testing for differences in concentrations of RANKL depending on the subject's race.

Test	F-ratio	df	P-value
Between Subjects			
Race	0.0438	1, 50	0.8351
Within Subjects			
Time	2.7744	4, 47	0.0377
Time-x-Race	0.3103	4, 47	0.8697

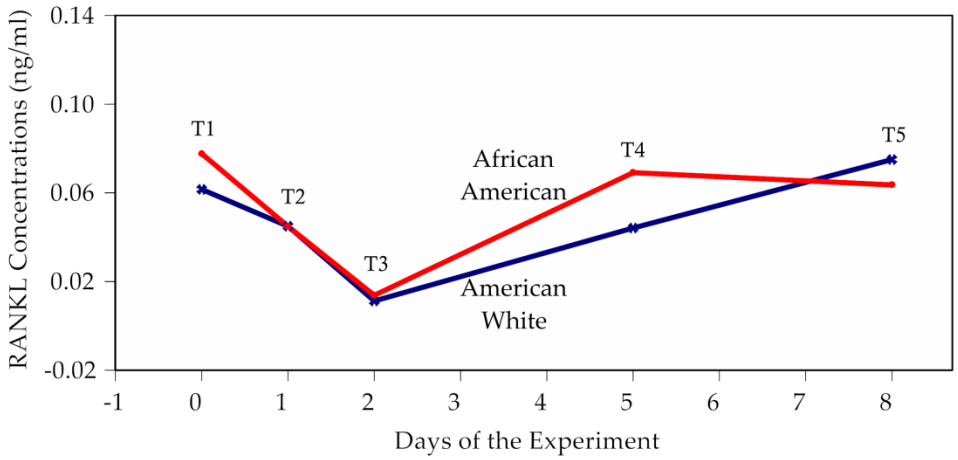

Figure 51. Graph of RANKL concentrations at the five examinations partitioned by subjects' race (American black or white).
RANKL values are the log-log transformations (plus 5). There was no statistically significant difference in concentrations between the two races (P = 0.8351).

Table 42. Descriptive statistics for RANKL concentrations partitioned by race.

Statistic	African American	American White
	log(RANKL) T1	
Mean	0.062	0.078
Std Deviation	0.157	0.160
Std Error of Mean	0.029	0.033
Upper 95% Mean	0.121	0.147
Lower 95% Mean	0.002	0.008
n	29	23
	log(RANKL) T2	
Mean	0.045	0.045
Std Deviation	0.147	0.139
Std Error of Mean	0.027	0.029
Upper 95% Mean	0.101	0.105
Lower 95% Mean	-0.011	-0.015
n	29	23
	log(RANKL) T3	
Mean	0.011	0.014
Std Deviation	0.134	0.144
Std Error of Mean	0.025	0.030
Upper 95% Mean	0.062	0.076
Lower 95% Mean	-0.040	-0.048
n	29	23
	log(RANKL) T4	
Mean	0.044	0.069
Std Deviation	0.128	0.141
Std Error Mean	0.024	0.029
Upper 95% Mean	0.093	0.130
Lower 95% Mean	-0.004	0.008
n	29	23
	log(RANKL) T5	
Mean	0.075	0.064
Std Deviation	0.146	0.153
Std Error of Mean	0.027	0.032
Upper 95% Mean	0.131	0.130
Lower 95% Mean	0.019	-0.003
n	29	23

Table 43. Results of MANOVA testing for differences in concentrations of OPG depending on whether the subject is American black or white.

Source	F-ratio	df	P-value
Between Subjects			
Race	1.39	1, 50	0.2440
Within Subjects			
Time	3.47	4, 47	**0.0145**
Time-x-Race	1.34	4, 47	0.2683

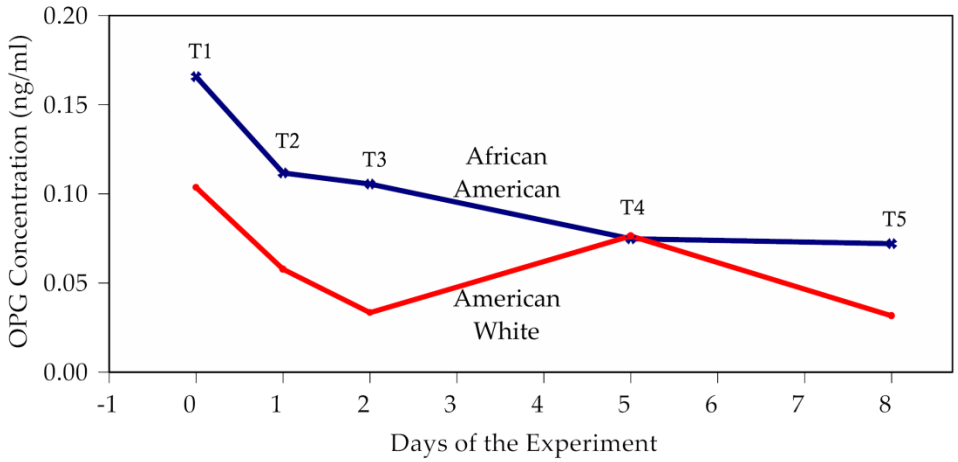

Figure 52. Plot of OPG concentrations at the five examinations, partitioned by the subject's race.
There was no statistically significant difference (P = 0.2440) by this grouping.

Table 44. Descriptive statistics for OPG concentrations partitioned by race.

Statistic	African American	American White
	loglog(OPG) T1	
Mean	0.166	0.104
Std Deviation	0.192	0.191
Std Error of Mean	0.036	0.040
Upper 95% Mean	0.239	0.186
Lower 95% Mean	0.093	0.021
n	29	23
	loglog(OPG) T2	
Mean	0.112	0.058
Std Deviation	0.155	0.174
Std Error of Mean	0.029	0.036
Upper 95% Mean	0.171	0.133
Lower 95% Mean	0.053	-0.018
n	29	23
	loglog(OPG) T3	
Mean	0.106	0.033
Std Deviation	0.167	0.156
Std Error of Mean	0.031	0.033
Upper 95% Mean	0.169	0.101
Lower 95% Mean	0.042	-0.034
n	29	23
	loglog(OPG) T4	
Mean	0.075	0.076
Std Deviation	0.148	0.155
Std Error of Mean	0.027	0.032
Upper 95% Mean	0.131	0.143
Lower 95% Mean	0.019	0.009
n	29	23
	loglog(OPG) T5	
Mean	0.072	0.032
Std Deviation	0.173	0.158
Std Error of Mean	0.032	0.033
Upper 95% Mean	0.138	0.100
Lower 95% Mean	0.006	-0.037
n	29	23

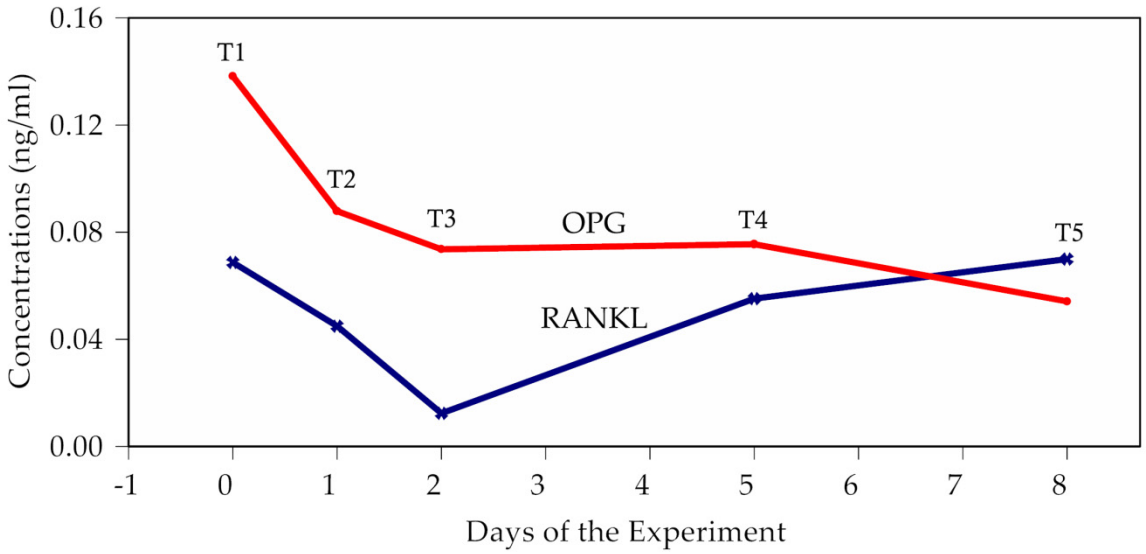

Figure 53. Plots for the concentrations of RANKL and OPG shown on the same graph.
Data were loglog transformed (plus 5).

concentrations are slow to return to equilibrium after spring removal (just 3 days). These data suggest that the RANKL/OPG ratio ought to change little until after day 2, when it appears that OPG becomes less common, which would increase the RANKL/OPG ratio.

Figure 54 is a plot of the RANKL/OPG ratio against time. For clarity, this ratio was calculated by first transforming the individual data and then taking the ratio:

$$\frac{\text{LogLog (RANKL + 5)}}{\text{LogLog (OPG + 5)}}$$

There is no apparent change from baseline to day 1, but then both molecules drop in concentration by day 2 (more so with OPG), and the ratio spikes dramatically at day 2. This erratic pattern is due to a few outliers at day 2.

Using the MANOVA test relied on before, there is no change among the five examinations ($F = 0.62$; $df = 4$ and 25; $P = 0.6514$), but this is distorted by the extreme sample variance at day 2. **Table 45** shows the attempt at making order of the data by applying Wilcoxon's signed rank tests to the 10 comparisons pairwise. The table lists the P-values, and none of the tests were significant.

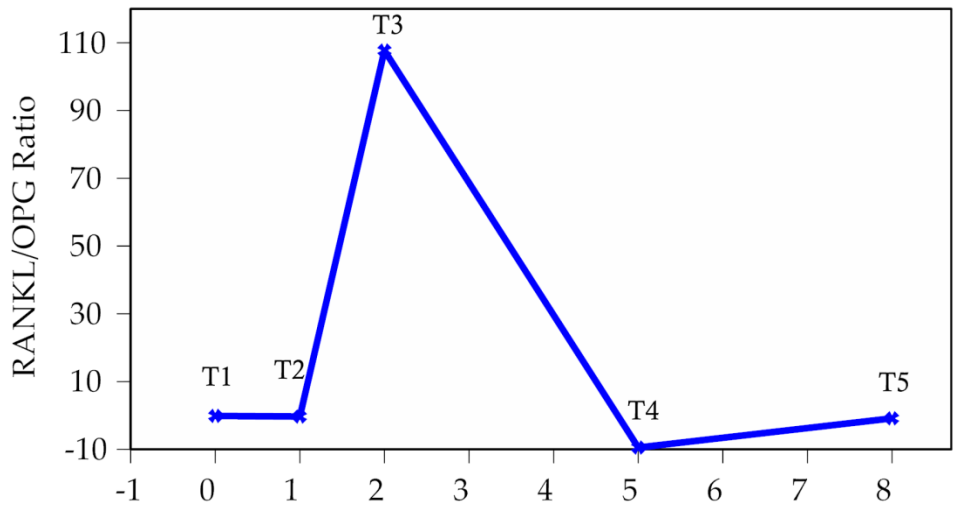

Figure 54. Plot of the RANKL/OPG ratio against time.

Table 45. Matrix of P-values from Wilcoxon signed-rank tests based on paired data between observations in the RANKL/OPG ratios.

	Group			
Group	T1	T2	T3	T4
T2	0.6896			
T3	0.9245	0.7280		
T4	0.9294	0.5330	0.8911	
T5	0.8074	0.8911	0.6050	0.6411

CHAPTER 5. DISCUSSION

Gingival Crevicular Fluid Volume

The present study was designed to evaluate changes in the expression of RANKL and OPG in gingival crevicular fluid (GCF) before, during, and after the application of an orthodontic force in both growing and non-growing humans. GCF samples were collected at the maxillary premolar sites at five different time points over an 8 day period (**Figure 5**). A baseline sample was collected prior to force application (T1); three samples were collected during force application (by a nickel titanium spring), namely at days 1, 2, and 5 (T2, T3, and T4 respectively); and a final sample was collected 3 days after force removal (T5).

Crevicular Fluid

Results show a significant increase in GCF levels for both the tension and compression sides following force application. GCF levels progressively increased from baseline (T1) at each time point (T2 through T4) until spring removal (T4). The buccal and lingual levels of GCF returned toward baseline after force removal; however, the lingual fluid levels at T5 were significantly higher than those at baseline (T1). The results of this study suggest that increased GCF volumes are related to orthodontic tooth movement.

Evaluation of the buccal and lingual GCF levels revealed a significantly higher amount of GCF on the lingual collection sites than on the buccal sites. On the buccal, the only source of significant increase in GCF was seen at T4. Conversely, a statistically significant increase was seen at every time point (T2, T3, and T4) for the lingual collection sites. The anatomical shape of each tooth and therefore its surrounding sulcus may partially be responsible for the differences seen in the GCF levels on each side of the tooth, but the applied mechanical force plays a significant role in the increased production of GCF.

Total GCF volumes increased significantly at each reading following force application (**Figure 10**); therefore force application increases the amount of GCF found in the sulcus. After force removal, the volumes of GCF significantly dropped, which is suggestive of the sulcus returning to its homeostatic state following the removal of a mechanical force. These results suggest that GCF quantities respond quickly to mechanical stress, and about as quickly to its removal.

Sequential increases in GCF volumes were seen during the period of force application. The tension of the spring was set in a range of 150 to 250 grams of force,

which is the range suggested by Storey and Smith (1952) for optimum tooth movement. Since a nickel titanium spring was used, an approximately constant force was delivered to the premolars over the 5-day period due to its superelastic properties (Muira *et al*. 1988). We speculate that a constant mechanical force results in an uninterrupted stimulus to the cells of the periodontal ligament (PDL) and alveolar bone, which then will produce a steady state of bone turn-over. If another material had been used, such as elastomeric chain or elastic rubber bands, the amount of GCF produced could be expected to decrease significantly over time due to force decay.

Previous reports regarding the effect of orthodontic tooth movement on the GCF have been conflicting. Tersin (1978) observed gingival exudation during orthodontic treatment, and concurrently he reported an increase in GCF volumes during mechanical force application. Baldwin *et al.* (1999) also reported an increase in GCF volume induced by orthodontic tooth movement. Yamaguchi *et al.* (2009) reported on differences in levels of GCF between conventional and Damon bracket systems. This study revealed that increases in GCF were less noticeable with the Damon bracket system, which the authors attributed to the passive ligation system with less inherent friction. Conversely, both Miyajima *et al.* (1991) and Uematsu *et al.* (1996) reported no statistical difference between the amount of fluid expressed around teeth undergoing orthodontic movement and control teeth. A current study investigating the change in volume of GCF exudate during canine retraction reported a slight, but non-significant, increase GCF levels (Dannan *et al.* 2009). Most recently, a study with similar methodology to ours reported a significant increase in GCF volumes during orthodontic force application, as well as a significant decrease towards baseline GCF levels following force removal (Hamman *et al.* 2009).

Demographic Predictors of Gingival Crevicular Fluid Volume

An aim of this study was to evaluate the effects of mechanical stress caused during tooth movement and its response in different ages, sexes, and races. Due to the different variables being tested and the available participant pool, it was impossible to recruit balanced and equal groups regarding sex, age, and race. A larger number of participants were enrolled in the study than previous studies to compensate for the sampling inequalities.

When looking at the buccal and lingual sites individually, no significant difference was found with regard to age, sex or race. However, adult participants showed a higher mean amount of GCF at all time points when compared to the adolescent group for the lingual collection sites. The increased lingual fluid amount was not significant ($P = 0.09$); however, it was highly suggestive. A study with a larger, more homogenous sample would help correct for the intra-group variability in this study. Interestingly, but not statistically significant, females had higher levels of GCF collected

from their lingual sites, while males, on the contrary, had higher levels of GCF collected from their buccal sites. The amount of GCF collected from both Caucasians and African Americans were nearly identical, and therefore not statistically significant. There is no reason to suspect that American blacks and whites would produce different GCF levels. The comparison was simply made because the comparison was available at our institution.

Some studies have reported a decrease in crevicular fluid flow in response to localized and systemic medication; however, these medications should not have impacted the results of our study due to the stringent selection criteria. Heasman and Seymour (1989) indicated that the NSAID flurbiprofen (Ansaid®) decreased the amount of crevicular fluid flow after 3 weeks of use. In 2006, a study by Eviö *et al.* reported that alendronate (Fosamax®), which is used in the treatment of bone loss associated with menopause, significantly decreased the crevicular fluid flow. Antibiotics, either topical or systemic, appear to have no affect on the gingival crevicular fluid flow (Asikainen *et al.* 1990; Ozcan *et al.* 2004; Pendrill and Reddy 1980; Tanner *et al.* 1994).

When evaluating the changes in gingival crevicular fluid in response to mechanical force, it appears that *time* is the significant factor regardless of the person's age, sex, or race. The limiting factor in the amount of GCF produced was dependent on the amount of time the spring was in place. Force from the spring increased the amount of crevicular fluid produced on both the buccal and lingual sides of the tooth. After the spring was removed, the stimulus for increased GCF was gone, and the fluid levels returned to near pre-treatment levels. The short timeline of the current study is a limitation, and a more in depth clinical study during actual orthodontic cases would reveal a broader, more realistic view of the changes in GCF levels during orthodontic treatment. An in-depth longitudinal study would reveal the point at which the GCF levels peak, flatten out, and then return to baseline.

RANKL and OPG Detectability

Gingival crevicular fluid is an exudate produced by the periodontal tissues. When the periodontium is healthy, only negligible amounts of gingival crevicular fluid is produced. On the other hand, production significantly increases in the presence of gingival inflammation. Research has shown that GCF production is increased in response to bacterial insult such as gingivitis (Brecx *et al*. 1987). During orthodontic tooth movement, however, only part of the increase in production of GCF can be attributed to inflammation caused by increased plaque retention (Samuels *et al*. 1993). It has also been reported that GCF production increases even when subjects undergo adequate plaque control (Tersin 1978). In the present study, the participants were required to perform adequate measures to prevent plaque retention around the spring

apparatus. By encouraging good plaque control standards, the present study tried to achieve a control mechanism for false positives in the collection of data.

The forces produced during mechanical tooth movement, as seen in orthodontics, will produce an acute generalized inflammatory reaction in the periodontal tissues (Kyrkanides *et al*. 2000). The immediate increase in the flow of gingival crevicular fluid is caused by the mechanical compression of the microvasculature of the periodontal ligament (PDL) resulting in an increased fluid volume in the crevicular space. This reaction causes areas of hyalinization to occur due to capillary constriction. Consequently, this chain of events leads to the initiation of an inflammatory reaction (Murrell *et al.* 1996). The release of a host of chemical mediators by the cells of the PDL in response to the areas of localized aseptic necrosis changes the composition of the GCF expressed into the gingival sulcus. This has been demonstrated by a number of studies where expression of pro-inflammatory cytokines in GCF tend to increase in response to mechanical forces (Dudic *et al*. 2006; Iwasaki *et al*. 2005; Rohaya *et al*. 2009; Uematsu *et al*. 1996). In the present study, the identification of RANKL and OPG were identified in the majority of the samples; however, there were still many instances in which the levels of RANKL and/or OPG were below the level of detectability for the sensitivity of the assays that were used.

In the few publications regarding the collection of GCF to examine RANKL and OPG, the results have been minimal and conflicting. There appears to be a significant problem in consistently detecting both RANKL and OPG from the samples of crevicular fluid. Bostanci *et al.* (2007) reported finding minimal levels of RANKL in patients with healthy gingiva and gingivitis, while finding OPG in all subjects. In contrast, Lu *et al.* (2006) did not find OPG in the GCF of any of his fourteen human subjects with healthy periodontium. Toygar (2008) *et al.* found OPG in the GCF of teeth undergoing orthodontic retraction and teeth not undergoing orthodontic mechanics. A recent study with a similar experimental protocol by Hamman *et al.* (2009) reported only 21.6% RANKL and 2.92% OPG detectability, meaning that most of the samples had concentrations below the level of detectability.

A likely explanation for the differences in cytokines detection is the differences in assay sensitivity among these different studies. In an attempt to overcome this limitation, several changes in protocol were introduced in this study. First, the perio paper strip was allowed to remain in the sulcus for a full minute to allow it to absorb as much GCF as possible. Also, the samples of the dropout participants were used to obtain a dilution factor in the laboratory that enabled a minimal amount of buffer to be used to extract the RANKL and OPG, which allowed detection of a maximum amount of RANKL and OPG with less background noise. The present study also incorporated sample "pooling" and duplicate measurements to attempt to increase the likelihood of achieving a detectable levels of RANKL and OPG. Technology in the development of

the assay kits most likely has improved over time as well, which allowed for a greater sensitivity and detectability of smaller amounts of RANKL and OPG.

Inevitably, when looking back at the current protocol, one can see certain areas that could be improved upon which might make the experiment more predictable. Primarily, a more efficient collection mechanism would allow for higher percentage of the GCF to be analyzed. Currently, the perio paper strips are the only dependable and measureable way to collect crevicular fluid. An inherent problem with the strips is the fluid and extraction buffer become trapped in the strip, which make it impossible to remove from the strip for the assaying procedures. Therefore, a significant amount of the sample plus buffer is lost. In order to achieve enough of a sample to assay, additional extraction buffer is added. The additional buffer possibly causes over-dilution of the sample, which leads to the levels of RANKL and OPG being below the level of detection of the assay. Consequently, if another reliable transport system is developed to correct for this problem, then it should be used.

RANKL and OPG Concentrations

To date, no publications are known on the *in vitro* evaluation of RANKL and OPG in response to mechanical forces in humans. Multiple studies exist on the evaluation of RANKL and OPG in rats and in extracted teeth. The present study evaluated the concentrations of both RANKL and OPG over time, and then evaluated the concentrations of these biomolecules with regard to three sources of variation, namely sex, age, and race.

The raw data results for both RANKL and OPG exhibited high levels of skewness. A log-log transformation was used to normalize these distributions, which allowed parametric statistical analysis. These transformations satisfactorily decreased the skewness and kurtosis, so the tests of the transformed distributions did not differ from normality.

RANKL and OPG Concentrations versus Time

As previously discussed, RANKL and OPG competitively regulate the body's bony metabolism. RANKL is a profound inducer of bone destruction, while OPG plays a reverse role to inhibit bony breakdown and serves as a protector of bone. RANK and OPG competitively bind to RANKL on preosteoclasts (Ikeda *et al*. 2001); consequently, whichever molecule (RANK or OPG) binds first will determine the fate of the surrounding bone.

RANKL expression by osteoblasts is required for osteoclastogenesis and for maturation of osteoclast precursor cells, and osteoblasts can also stimulate rapid bone resorption by activating pre-existing osteoclasts (Boyle *et al.* 2003). RANKL partially regulates the survival of mature osteoclasts by increasing their mean survival time (Feige 2001; Suda *et al.* 1999). However, RANKL is not required for mature osteoclast function, which can be regulated by other cytokines such as M-CSF and TRAF6. After RANKL stimulates osteoclast formation and activation, their continued function depends on other cellular messages received by cell to cell interaction via cytokines with osteoblasts (Arai *et al.* 1999; Burgess *et al.* 1999). Therefore, peak osteoclastic activity may not coincide with the peak RANKL expression. The increase in RANKL expression by osteoblasts may occur much sooner after the initiation of orthodontic force.

OPG competitively inhibits and reversibly binds to the RANKL site of preosteoclasts (Yasuda *et al.* 1998). This competitive, yet reversible, binding inhibits osteoclastogenesis by terminating their differentiation into mature osteoblasts (Kanzaki *et al.* 2005). OPG has profound inhibitory effects on osteoclast differentiation and bone resorption, and its full name, osteoprotegerin, literally means protector of bone (Liebbrandt and Penninger 2008). OPG expression is regulated by the same hormones and proteins found naturally in the body that also are influential in the body's bony metabolism (Horowitz *et al* 2001).

In this study, RANKL concentrations decreased significantly over the time the spring was in place. Unexpectedly, the levels of RANKL were lowest at T3, or 48 hours after spring activation. RANKL acted contrary to expectations by decreasing from baseline with an increased time of force application, then rebounding toward baseline after the removal of the force. The amount of RANKL should have increased in a time-dependent manner following force application due to the increased osteoclastogenesis induced by the transpalatal spring. However, the increase in the RANKL/OPG ratio is also accomplished by a decrease in OPG concentrations, while the RANKL concentrations remain constant. The end result would be the same for either scenario meaning higher amounts of free RANKL, therefore more osteoclast stimulation. The OPG concentrations decreased steadily over time through the duration of the experiment. OPG levels were highest at baseline, and they did not begin to return to baseline following force removal. OPG's behavior seen in this study is to be expected, since the mechanical force delivered by the spring encourages bony remodeling (**Figure 53**).

The findings in this study for RANKL concentration over time are contradictory to previous studies. Brooks *et al*. (2009) reported that increases in RANKL can be seen as early as 24 hours following a constant orthodontic force. According to Li *et al.* (2011), heavier forces produce concentrations of RANKL over an extended period of time when compared to a light force. Reports by Kim *et al.* (2007) stated that PDL cells are constantly producing RANKL during continuous orthodontic force. However, there

were not an excessive number of osteoclasts produced. On the other hand, the decreased levels of OPG after force application seen in this study are supported by several prior studies. Toygar *et al*. (2008) reported OPG concentration decrease in a time-dependent manner from baseline, and he concluded that OPG is one of the key mediators in alveolar bone remodeling during orthodontic tooth movement. In 2006, Nishijima *et al*. demonstrated that OPG levels were significantly decreased, while RANKL levels were significantly increased at 24 hours after force application.

The departure of RANKL concentrations from the expected pattern could be explained by the specificity of the ELISA assay used for this experiment. The assay utilized in this study only detects the soluble isoform of RANKL. Also, the system used was cell-free; as a result, no cell-bound RANKL was available to be detected. Therefore, if the majority of the RANKL produced during orthodontic force is one of the transmembrane isotypes, our assay would not have the capability of detecting it. As technology advances, the assay kits will most likely become more sensitive to detecting these biomolecules in their different isoforms. In addition, our analysis of RANKL was purely extracted from human gingival crevicular fluid without using invasive measures. RANKL and OPG can also be made by PDL fibroblasts, gingival fibroblasts, and lymphocytes. Therefore, it is possible that substantial amounts of RANKL reside in the PDL tissues and not the crevicular fluid itself. To date, most studies involving orthodontic forces and the production of RANKL and OPG involve rats, beagles, and the extracted human premolar teeth. In these studies, the PDL tissue can be examined more closely under immunofluorescence or histologically. By merely looking at the collections of gingival crevicular fluid, this study was limited to the expression of RANKL and OPG found freely moving about the gingival sulcus.

RANKL: Sources of Variation

When evaluating the RANKL in regards to sex, the literature falls short. Currently, there is no published study that discusses the levels of RANKL in males versus females in regards to orthodontic tooth movement. The results of the present study suggest that there is no significant difference in RANKL between men and women undergoing orthodontic tooth movement. However, males consistently had greater levels of RANKL than females at each examination time (**Figure 36**).

Kawasaki *et al.* (2006) showed that the degree of tooth movement is greater in children than in adults when exposed to the same amount and duration of orthodontic force. Hence, one might conclude that children have a higher level of RANKL in their crevicular fluid, while adults have a lower level of RANKL in their crevicular fluid. For RANKL, the results of our study contradict those findings. This study revealed that there is no difference in RANKL expression between adults and adolescence when exposed to a mechanical orthodontic force (**Figure 43**).

RANKL levels were also evaluated in American whites and African Americans. This aspect of the study was added due to sampling convenience in the patient population. No other studies can be found which evaluates RANKL levels between the two populations. For this study, no significant difference was found between the two races in RANKL levels.

OPG: Sources of Variation

From an in depth review of the literature, there appears to be no studies published on sex difference in the levels of OPG during orthodontic force application. The results of our study show a significantly higher amount of OPG in males at all five time points. The source of the significance between the sexes is two-fold: 1) females had an increased level of RANKL at T5, and 2) males had an increased level of OPG at baseline. The difference in the baseline measurements between males versus females is an interesting finding. It appears that women have less OPG in general, which might explain why women are more susceptible to osteoporosis than their male counterparts (**Figure 37**).

As mentioned, Kawasaki *et al.* (2006) reported that adults have less clinical tooth movement than adolescence, so it can be surmised that adults have more OPG than children. The present study showed that adults have less OPG than their younger counterparts. The decrease in OPG levels appears to be linear with age, though there is a lot of inter-individual variability (**Figure 49**). It is possible that the younger subjects had increased amounts of OPG due to the constant bony deposition associated with growth. However, if normal growth and development causes an increase in OPG levels in a growing child, then one must also assume that RANKL levels should be increased as well, which is not the case. A future study might shed light on the individual changes in the RANKL/OPG ratio differences between adults and children over time in response to a mechanical force.

This study was the first to look at OPG concentrations between American whites and African Americans in response to an orthodontic force. The data did not produce a significant difference in OPG levels between the races, however the OPG concentrations of the blacks were found to be higher at each examination than the whites (**Figure 52**). A larger, more homogenous sample might produce a significant difference. Clinically, tooth movement in African Americans is more difficult to achieve than in whites, especially with increased age. The higher levels of OPG found in blacks in this study might partially explain the increased difficulty of tooth movement in this race.

The RANKL/OPG Ratio

The RANKL/OPG ratio describes the relative state of the body's bony metabolism. This ratio expresses the existing state of the bone's regulatory system, which is controlled by the regulation of specific gene expression. The RANKL/OPG regulatory axis serves the purpose of maintaining bone structure and function, in addition to meeting the body's physiological needs for ions sequestered in bone (Boyle *et al.* 2003; Kanzaki *et al.* 2001). An increased RANKL/OPG ratio would favor osteoclast formation and activation; as a result, bone resorption occurs. On the other hand, a decreased RANKL/OPG ratio promotes bone formation by inhibiting osteoclastic activity (Kanzaki *et al.* 2001). An increase in RANKL and a decrease in OPG during osteoclastogenesis is reported in the literature (Kanzaki *et al.* 2001; Liebbrandt and Penninger 2008). Thus, an increase in the RANKL/OPG ratio should be seen with continuous mechanical force over time. In this study, OPG decreased with increased duration of force; however the levels of OPG did not rebound after force removal. For RANKL, the levels initially dropped, but then increased two days after spring placement. After the removal of the spring, the RANKL levels returned toward baseline. The data from this study suggest that the RANKL/OPG ratio changes little after day 2 (48 hours after spring placement), when OPG becomes less common, which in effect increases the RANKL/OPG ratio. A future study with increased precision and better assay sensitivity will allow a more in depth examination of the changes in the RANKL/OPG ratio over time in response to a mechanical force. A disadvantage to this study was the fair amount of "zeros" reported in the assays. The zero value does not mean that RANKL or OPG was not present, but unfortunately that the levels of these biomolecules were below the level of detectability of the assay.

CHAPTER 6. SUMMARY AND CONCLUSIONS

This study evaluated changes in RANKL and OPG expression in human gingival crevicular fluid in growing (adolescent) and non-growing (adult) patients in response to orthodontic force. This study assessed 54 participants of various ages. Although there were no set age groupings for this study, in order to treat age as a category, a number of the patients were less than 16 years of age or older than 30 years. The adolescent and adult groups were matched as best as possible for age, sex, and race. Major finding included:

- The volume of GCF expressed into the sulcus significantly increased following application of orthodontic force and remained elevated until after the removal of the force. This supports the theory that orthodontic tooth movement is associated with an inflammatory-like reaction of the periodontium in response to mechanical force.
- When comparing buccal and lingual GCF production, higher amounts of GCF were seen in the lingual sites throughout the duration of the study.
- GCF expression exhibits high inter-individual variability.
- There was no statistically significantly differences found in the levels of GCF when comparing age, sex, and race; however, some of the findings were highly suggestive:
    - Adult participants showed a higher mean amount of GCF at all time points when compared to the adolescent group for the lingual collection sites.
    - Females had higher levels of GCF collected from their lingual sites, while males, on the contrary, had higher levels of GCF collected from their buccal sites.
    - The amount of GCF collected from both Caucasians and African Americans were nearly identical, and therefore not statistically significant.
- When evaluating the changes in gingival crevicular fluid in response to mechanical force, it appears that *time* is the significant factor regardless of the person's age, sex, or race.
- The identification of RANKL and OPG were identified in the majority of the samples; however, there were still many instances in which the levels of RANKL and/or OPG were below the level of detectability for the sensitivity of the assays that were used.
- Whereas RANKL and OPG are readily detectable in GCF obtained from patients with periodontitis, these cytokines appear to be expressed in smaller amounts in response to orthodontic force.
- RANKL concentrations decreased significantly over the time the spring was in place.

- The OPG concentrations decreased steadily over time through the duration of the experiment.
- The RANKL/OPG ratio increased over time, which favors osteoclast activation and formation leading to bone resorption.
- No significant differences were found in the levels of RANKL when comparing sex, age, and race. However, males consistently had greater levels of RANKL than females at each examination time.
- Significantly higher levels of OPG were found in males throughout the duration of the study.
- Adults have less OPG than their younger counterparts. The decrease in OPG levels appears to be linear with age, though there is a lot of inter-individual variability.
- The OPG concentrations of the African Americans were found to be higher at each examination than the Caucasians; but it is not statistically significant.
- RANKL and OPG may be present in greater levels in the tissues surrounding the teeth. The levels present in the GCF may not be representative of what is occurring in the tissues during orthodontic tooth movement.
- Further research is needed using more sensitive methods to better describe the changes in RANKL and OPG in response to orthodontic force, and what effects these molecules have on orthodontic tooth movement.

LIST OF REFERENCES

Abbott B, Caffesse R. Crevicular fluid: origin, composition, methods of collection, and clinical significance. J West Soc Periodontol Periodont Abstr 1977;25:164-78.

Alfano MC. The origin of gingival fluid. J Theor Biol 1974;47:127-36.

Al-Qawasmi RA, Hartsfield JK Jr, Everett ET, Weaver MR, Foroud TM, Faust DM, Roberts WE. Root resorption associated with orthodontic force in inbred mice: genetic contributions. Eur J Orthod 2006;28:13-9.

Anandarajah AP. Role of RANKL in bone diseases. Trends in Endocrinology and Metabolism 2009;20:88-94.

Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. J Exp Med 1999;190:1741-54.

Arias OR, Marquez-Orozco MC. Aspirin, acetaminophen, and ibuprofen: their effects of orthodontic tooth movement. Am J Orthod Dentofacial Orthop 2006;130:364-70.

Asikainen S, Jousimies-Somer H, Kanervo A, Saxén L. The immediate efficacy of adjunctive doxycycline in treatment of localized juvenile periodontitis. Arch Oral Biol 1995;35:231S-34S.

Baldwin PD, Pender N, Last KS. Effects on tooth movement of force delivery from nickel-titanium archwires. Eur J Orthod 1999;21:481-9.

Basset CAL, Becker RO. Generation of electric potentials by bone in response to mechanical stress. Science 1962;137:1063-5.

Baumrind S. A reconsideration of the propriety of the “pressure-tension ” hypothesis. Am J Orthod 1969;55:12-22.

Bostanci N, Ilgenli N, Emingil G, Afacan B, Han B, Töz H, Atilla G, Hughes FJ, Belibasakis GN. Gingival crevicular fluid levels of RANKL and OPG in periodontal diseases: implications of their relative ratio. J Clin Periodontol 2007;34:370-6.

Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature 2003;423:337-42.

Brecx MC, Schlegel K, Gehr P, Lang MP. Comparison between histological and clinical parameters during human experimental gingivitis. J Periodont Res 1987;22:50-7.

Brill n. The gingival pocket fluid. Studies of its occurrence, composition, and effect. Acta Odont Scand1969;20(suppl 32):159.

Brooks PJ, Nilforoushan D, Manolson MF, Simmons CA, Gong SG. Molecular markers of early orthodontic tooth movement. Angle Orthod 2009;79:1108-13.

Buduneli n, Biyikoglo S, Sherrabeh S, Lappin DF. Saliva concentrations of RANKL and osteoprotegerin in smoker versus non-smoker chronic periodontitis patients. J Clin Periodontol 2008;35:846-52.

Burgess TL, Qian Y, Kaufman S, Ring BD, Van G, Capparelli C, Kelley M, Hsu H, Boyle WJ, Dunstan CR, Hu S, Lacey DL. The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. J Cell Biol 1999;145:527-38.

Carranza FA, Newman MG, editors. Clinical Periodontology. Philadelphia: WB Saunders 1996;8:103-9.

Chutimanutskul W, Darendeliler MA, Shen G, Petocz P, Swain MV. Changes in the physical properties of human premolar cementum after application of 4 weeks of controlled orthodontic forces. Eur J Orthod 2006;28:313-8.

Dannan A, Darwish MA, Sawan MN. Effect of orthodontic tooth movement on gingival crevicular fluid infiltration; a preliminary investigation. J Dent 2009;6:109-15.

Delima AJ, Van Dyke TE. Origin and function of the cellular components in gingival crevice fluid. Periodontol 2000. 2003;31:55-76.

Dudic A, Kiliaridis S, Mombelli A, Giannopoulou C. Composition changes in gingival crevicular fluid during orthodontic tooth movement: comparisons between tension and compression sides. Eur J Oral Sci 2006;114:416-22.

Eviö S, Tarkkila L, Sorsa T, Furuholm J, Välimäki MJ, Ylikorkala O, Tiitinen A, Meurman JH. Effects of alendronate and hormone replacement therapy, alone and in combination, on saliva, periodontal conditions and gingival crevicular fluid matrix metalloproteinase-8 levels in women with osteoporosis. Oral Dis 2006;12:187-93.

Feige U. Osteoprotegerin. Ann Rheum Dis 2001;60:81-4.

Freund RJ, Littell RC. SAS® system for regression, 2nd ed. Cary, NC: SAS Institute Inc, 1991.

Frost HM. Presence of microscopic cracks *in vivo* in bone. Henry Ford Hospital Medical Bulletin 1960;8:25-35.

George A, Evans CA. Detection of root resorption using dentin and bone markers. Orthod Craniofac Res 2009; 12:229-35.

Gianelly AA. Force induced changes in the vascularity of the periodontal ligament. Am J Orthod 1969;55:5-11.

Gillooly CJ, Hosley RT, Mathews JR, Jewett DL. Electrical potentials recorded from mandibular alveolar bone as a result of forces applied to the tooth. Am J Orthod 1968;54:649-54.

Graber TM, Vanarsdall RL, Vig KWL, editors. Orthodontics. Current Principles and Techniques, 4th Edition. St Louis: Mosby Elsevier 2005.

Grimm FM. Bone bending, a feature of orthodontic tooth movement. Am J Orthod 1972;62:384-93.

Hamman NR. Modulation of RANKL and Osteoprotegerin in Adolescents Using Orthodontic Forces. Master's Thesis, University of Tennessee, May 2009.

Harokopakis-Hajishengallis E. Physiologic root resorption in primary teeth: molecular and histological events. J Oral Sci 2007;49:1-12.

Harrell A, Dekel S, Binderman I. Biochemical effect of mechanical stress on cultured bone cells. Calcified Tissue Research 1977;22:202-7.

Harris EF. External apical root resorption resulting from orthodontic tooth movement. Semin Orthod 2000;6:183-94.

Harris EF, Kineret SE, Tolley EA. A heritable component for external apical root resorption in patients treated orthodontically. Am J Orthod Dentofacial Orthop 1997;111:301-9.

Hartsfield JK, Everett ET, Al-Qawasmi RA. Genetic factors in external apical root resorption and orthodontic treatment. Crit Rev Oral Biol Med 2004;15:115-22.

Heasman PA, Seymour RA. The effect of a systemically-administered non-steroidal anti-inflammatory drug (flurbiprofen) on experimental gingivitis in humans. J Clin Periodontol 1989;16:551-6.

Heinrich J, Bsoul S, Barnes J, Woodruff K, Abboud S. CSF-1, RANKL and OPG regulate osteoclastogenesis during murine tooth eruption. Arch Oral Biol 2005;50:897-908.

Heller IJ, Nanda R. Effect of metabolic alteration of periodontal fibers on orthodontic tooth movement. An experimental study. Am J Orthod 1979;75:239-58.

Henneman S, Von den Hoff JW, Maltha JC. Mechanobiology of tooth movement. Eur J Orthod 2008;30:299-306.

Horowitz MC, Yougen X, Wilson K, Kacena MA. Control of osteoclastogenesis and bone resorption by members of the TNF family of receptors and ligands. Cytokine Growth Factor Rev 2001;12:9-18.

Ikeda T, Kasai M, Utsuyama M, Hirokawa K. Determination of three isoforms of the receptor activator of nuclear factor-kappaB ligand and their differential expression in bone and thymus. Endocrinology 2001;142:1419-26.

Iwasaki LR, Crouch LD, Tutor A, Gibson S, Hukmani n, Marx DB, Nickel JC. Tooth movement and cytokines in gingival crevicular fluid and whole blood in growing and adult subject. Am J Orthod Dentofacial Orthop 2005;128:483-91.

Jones DH, Kong YY, Penninger JM. Role of RANKL and RANK in bone loss and arthritis. Ann Rheum Dis 2002;61:32-9.

Kanzaki H, Chiba M, Arai K, Takahashi I, Haruyama n, Nishimura M, Mitani H. Local RANKL gene transfer to the periodontal tissue accelerates orthodontic tooth movement. Gene Therapy 2006;13:678-85.

Kanzaki H, Chiba M, Shimizu Y, Mitani H. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. J Dent Res 2001;80:887-91.

Kanzaki H, Chiba M, Shimizu Y, Mitani H. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappa B ligand up-regulation via prostaglandin E2 synthesis. J Bone Miner Res 2002;17:210-20.

Kanzaki H, Chiba M, Takahashi I, Haruyama N, Nishimura M, Mitani H. Local OPG gene transfer to periodontal tissue inhibits orthodontic tooth movement. J Dent Res 2004;83:920-5.

Kawasaki K, Takahashi T, Yamaguchi M, Kasai K. Effects of aging on RANKL and OPG levels in gingival crevicular fluid during orthodontic tooth movement. Orthod Craniofacial Res 2006;9:137-42.

Kim T, Handa A, Iida J, Yoshida S. RANKL expression in rat periodontal ligament subjected to a continuous orthodontic force. Arch Oral Biol 2007;52:244-50.

Kohno T, Matsumoto Y, Kanno Z, Warita H, Soma K. Experimental tooth movement under light orthodontic forces: rates of tooth movement and changes of the periodontium. J Orthod 2002;29:129-35.

Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Boyle WJ, Penninger JM. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature 1999;397:315-23.

Kowashi Y, Jaccard F, Cimasoni G. Sulcular polymorphonuclear leukocytes and gingival exudates during experimental gingivitis in man. J Periodontal Res 1980;15:151-8.

Krishnan V, Davidovitch Z. Cellular, molecular, and tissue-level reactions to orthodontic force. Am J Orthod Dentofacial Orthop 2006;129:469.e1-469.e32.

Krishnan V, Davidovitch Z. On a path to unfolding the biological mechanisms of orthodontic tooth movement. J Dent Res 2009; 88:597-608.

Kyrkanides S, O'Banion MK, Subtelny JD. Nonsteroidal anti-inflammatory drugs in orthodontic tooth movement: metalloproteinase activity and collagen synthesis by endothelial cells. Am J Orthod Dentofacial Orthop 2000;118:203-9.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins n, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998;93:165-76.

Lappin DF, Sherrabeh S, Jenkins WMM, Macpherson LMD. Effect of smoking on serum RANKL and OPG in sex, age, and clinically matched supportive-therapy periodontitis patients. J Clin Periodontol 2007;34:271-7.

Leenheer ED, Mueller GS, Vanderkerken K, Croucher PI. Evidence of a role for RANKL in the development of myeloma bone disease. Curr Opin Pharmacol 2004;4:340-6.

Li Y, Zheng W, Liu JS, Wang J, Yang P, Li ML, Zhao ZH. Expression of osteoclastogenesis inducers in a tissue model of periodontal ligament under compression. J Dent Res 2001;90:115-20.

Liebbrandt A, Penninger JM. RANK/RANKL: Regulators of immune responses and bone physiology. Ann NY Acad Sci 2008;1143:123-50.

Liebbrandt A, Penninger JM. RANK(L) as a key target for controlling bone loss. Adv Exp Med Biol 2009;647:130-45.

Liebbrandt A, Penninger JM. RANKL/RANK as key factors for osteoclast development and bone loss in arthropathies. Adv Exp Med Biol 2009;649:100-13.

Lin JM, Callon KE, Lin CQ, Bava U, Zheng MH, Reid IR, Cornish J. Alteration of bone cell function by RANKL and OPG in different *in vitro* models. Eur J Clin Invest 2007;37:407-15.

Littell RC, Henry PR, Ammerman CB. Statistical analysis of repeated measures data using SAS procedures. J Anim Sci 1998;76:1216-31.

Liu D, Yao S, Pan F, Wise E. Chronology and regulation of gene expression of RANKL in the rat dental follicle. Eur J Oral Sci 2005;113:404-9.

Liu L, Igarashi K, Kanzaki H, Chiba M, Shinoda H, and Mitani H. Clodronate inhibits PGE_2 production in compressed periodontal ligament cells. J Dent Res 2006; 85:757-60.

Löe H. Holm-Pederson P. Absence and presence of fluid from normal and inflamed gingiva. Periodontics 1965;3:171.

Low E, Zoellner H, Kharbanda OP, Darendeliler MA. Expression of mRNA for osteoprotegerin and receptor activator of nuclear factor kappa β ligand (RANKL) during root resorption induced by the application of heavy orthodontic forces on rat molars. Am J Orthod Dentofacial Orthop 2005;128:497-503.

Lu HK, Chen YL, Chang HC, Li CL, Kuo MY. Identification of the osteoprotegerin/receptor activator of nuclear facto-kappa B ligand system in gingival crevicular fluid and tissue of patients with chronic periodontitis. J Periodontal Res 2006;41:354-60.

Massella RS, Meister M. Current concepts in the biology of orthodontic tooth movement. Am J Orthod Dentofacial Orthop 2006;129:458-68.

Meikle MC. The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Stanstedt. Eur J Orthod 2006;28:221-40.

Meikle MC. Remodeling the dentofacial skeleton: the biological basis of orthodontics and dentofacial orthopedics. J Dent Res 2007;86:12-24.

Melsen B. Biological reaction of alveolar bone to orthodontic tooth movement. Angle Orthod 1999;69:151-8.

Menezes R, Garlet TP, Letra A, Bramante CM, Campanelli AP, Figueira RC, Sogayar MC, Granjeiro JM, Garket GP. Differential patterns of receptor activator of nuclear factor kappa B ligand/osteoprotegerin expression in human periapical granulomas: possible association with progressive or stable nature of the lesions. J Endod 2008;34:932-8

Miyajima K, Ohno Y, Iwata T, Tanida K, Iizuka T. The lactic acid and citric acid content in the gingival fluid of orthodontic patients. Aichi Gakuin Dent Sci 1991;4:75-82

Mogi M, Otogoto J, Ota n, Togari A. Differential expression of RANKL and osteoprotegerin in gingival crevicular fluid of patients with periodontitis. J Dent Res 2004;83:166-9.

Muira F, Mogi, M, Ohura Y, Karibe M. The super-elastic Japanese NiTI alloy wire for use in orthodontics. Part III. Studies on the Japanese NiTi alloy coil springs. Am J Orthod 1988;94:89-96.

Murrell EF, Yen EH, Johnson RB. Vascular changes in the periodontal ligament after removal of orthodontic forces. Am J Orthod Dentofacial Orthop 1996;110:280-6.

Nishijima Y, Yamaguchi M, Kojima T, Aihara n, Nakajima R, Kasal K. Levels of RANKL and OPG in gingival crevicular fluid during orthodontic tooth movement and effect of compression forces on releases from periodontal ligament cells *in vitro*. Orthod Cranio Res 2006;9:63-70.

Noda K, Nakamura Y, Oikawa T, Shimpo S, Kogure K, Hirashita A. A new idea and method of tooth movement using a ratchet bracket. Eur J Orthod 2007;29:225-31.

Ohazama A, Courtney JM, Sharpe PT. OPG, RANKL, and RANK in tooth development: co-ordination of odontogenesis and osteogenesis. J Dent Res 2004;88:241-4.

Oka H, Miyauchi M, Sakamoto K, Moriwaki S, Niida S, Noguchi K, Somerman MJ, Takata T. PG_{E2} activates cementoclastogenesis by cementoblasts *via* EP4. J Dent Res 2007;86:974-9.

Oppenheim A. Tissue changes, particularly of the bone, incident to tooth movement. American Orthodontist Volume III 1911, 1912:56-67,113-32.

Oppenheim A. Human tissue response to orthodontic intervention of short and long duration. Am J Orthod 1942;28:263-301.

Oshiro T, Shiotani A, Shibasaki Y, Sasaki T. Osteoclast induction in periodontal tissue during experimental movement of incisors in osteoprotegerin-deficient mice. Anat Rec 2002;266:218-25.

Ozcan G, Taner IL, Doğanay T, Iscanoğlu M, Taplamacioğlu B, Gültekin SE, Baloş K. Use of membranes containing 20% chlorhexidine and 40% doxycycline for treatment of chronic periodontal pockets. J Nihon Univ Sch Dent 1994;36:191-8.

Parker RJ, Harris EF. Directions of orthodontic tooth movements associated with external apical root resorption of the maxillary central incisor. Am J Orthod Dentofacial Orthop 1998;114:677-83.

Parris WG, Tanzer FS, Fridland GH, Harris EF, Killmar J, Desiderio DM. Effects of orthodontic force on methionine enkephalin and substance P concentrations in human pulpal tissue. Am J Orthod Dentofacial Orthop 1989;95:479-89.

Pendrill K, Reddy J. The use of prophylactic penicillin in periodontal surgery. J Periodontol 1980;51:44-8.

Proffit WR, Fields HW, Sarver DM, editors. Contemporary Orthodontics. St Louis: Mosby Elsevier 2007;4:331-58.

Reitan K. Tissue behavior during orthodontic tooth movement. Am J Orthod 1960;46:881-900.

Reitan K. Effects of force magnitude and direction of tooth movement of different alveolar types. Angle Orthod 1964;34:244-55.

Reitan K. Clincal and histologic observations on tooth movement during and after orthodontic treatment. Am J Orthod 1967;53:721-45.

Reitan K, Rygh P. Biomechanical principles and reactions. In: Graber TM, Vanarsdall RL, editors. Orthodontics: current principles and techniques. St Louis: CV Mosby 2005;4:145-292.

Ren Y, Maltha J, Kuijpers-Jagtman AM. Optimum force magnitude for orthodontic tooth movement: A systematic literature review. Angle Orthod 2003;73:86-92.

Rifkin BR, Gay CV, editors. Biology and physiology of the osteoclasts. Boca Raton: CRC Press, 1992.

Roberts WE, Ferguson DJ. Cell kinetics of periodontal ligament. In: Norton LA, Burstone CJ, editors. Biology of tooth movement. Boca Raton: CRC Press, 1995.

Roberts WE, Goodwin WC Jr, Heiner SR. Cellular response to orthodontic force. Dent Clin North Am 1981;25:3-17.

Roberts WE, Huja S, Roberts JA. Bone modeling—biomechanics, molecular mechanisms and clinical perspectives. Semin Orthod 2004;10:123-61.

Roberts-Harry D, Sandy J. Orthodontics. Part 11: Orthodontic tooth movement. Br Dent J 2004;196:391-4.

Roche JJ, Ciscernos GJ, Acs G. The effect of acetaminophen on tooth movement in rats. Angle Orthod 1997;67:231-6.

Rohaya MA, Shahrul Hisham ZA, Khazlina K. Preliminary study of aspartate aminotransferase activity in gingival crevicular fluids during orthodontic tooth movement. J Appl Sci 2009;9:1393-6.

Sahara n. Cellular events at the onset of physiological root resorption in rabbit deciduous teeth. Anat Rec 2001;264:387-96.

Samuels RH, Pender N, Last KS. The effects of orthodontic tooth movement on the glycosaminoglycan components of gingival crevicular fluid. J Clin Periodontol 1993;20:371-7.

Sasaki T. Differentiation and functions of osteoclasts and odontoclasts in mineralized tissue resorption. Microscopy Res Technique 2003;61:483-95.

Schwarz AM. Tissue changes incident to orthodontic tooth movement. Int J Orthod 1932;18:331-52.

Siegel S, Castellan NJ. Nonparametric statistics for the behavioral sciences, 2nd ed. New York: McGraw-Hill, Inc., 1988.

Sokal RR, Rohlf FJ. Biometry: the principles and practice of statistics in biological research, 3rd ed. San Francisco: WH Freeman and Company, 1995.

Stanstedt C. Einige Beiträge zur Theorie der Zahnregulierung. Nordisk Tandläkare Tidskrift 1904;5:236-56.

Storey, E. The nature of tooth movement. Am J Orthod 1973;63:292-314.

Storey E, Smith R. Force in orthodontics and its relation to tooth movement. Aust J Dent 1952;56:11–8.

Suda T, Takahashi n, Udagawa n, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocrine Reviews 1999;20:345-57.

Tang TH, Fitzsimmons TR, Bartold PM. Effect of smoking on concentrations of receptor activator of nuclear factor κ β ligand and osteoprotegerin in human gingival crevicular fluid. J Clin Periodontol 2009;36:713-8.

Tanner IL, Ozcan G, Doğanay T, Iscanolu M, Taplamacioğlu B, Gültekin SE, Baloş K. Comparison of the antibacterial effects on subgingival microflora of two different resorbable base materials containing doxycycline. J Nihon Univ Sch Dent 1994;36:183-90.

Tersin J. Studies of gingival conditions in relation to orthodontic treatment. IV. The effect of oral hygiene measures on gingival exudation during the course of orthodontic treatment. Swed Dent J 1978;2:131-6.

Toygar HU, Kircelli BH, Bulut S, Sezgin N, Tasdelen B. Osteoprotegerin in gingival crevicular fluid under long-term continuous orthodontic force application. Angle Orthod 2008;78:988-93.

Tryovola JB, Spyropoulos MN, Makou M, Perrea D. Root resorption and the OPG/RANKL/RANK system: a mini review. J Oral Sci 2008;50:367-76.

Tukey JW. Exploratory data analysis. Reading, Mass: Addision-Wesley, 1977.

Uematsu S, Mogi M, Deguchi T. Interleukin (IL)-1 beta, IL-6, tumor necrosis factor-alpha, epidermal growth factor, and beta 2-microglobulin levels are elevated in gingival crevicular fluid during human orthodontic tooth movment. J Dent Res 1996;75:562-7.

Vega D, Maalouf NM, Sakhaee K. The role of RANK/RANK ligand/OPG: clinical implications. J Clin Endocrinol Metab 2007;92:4514-21.

Verna C, Dalstra M, Lee TC, Cattaneo PM, Melsen B. Microcracks in the alveolar bone following orthodontic tooth movement: a morphological and morphometric study. Eur J Orthod 2004;26:459-67.

Vitovski, S, Phillips, JS, Sayers, J, Croucher, PI. Investigating the interaction between osteoprotegerin and RANKL or TRAIL: Evidence for a pivotal role for osteoprotegerin in regulating two distinct pathways. J Biol Chem 2007;282:31601-9.

Wise GE, King GJ. Mechansims of tooth eruption and orthodontic tooth movement. J Dent Res 2008;87:414-34.

Yamaguchi M. RANK/RANKL/OPG in orthodontic tooth movement. Orthod Craniofac Res 2009;12:113-9.

Yamaguchi M, Aihara n, Kojima T, Kasai K. RANKL increase in compressed periodontal ligament cells from root resorption. J Dent Res 2006;85:751-6.

Yamaguchi M, Takizawa T, Nakajima R, Imamura R, Kasai K. The Damon system and release of substance P in gingival crevicular fluid during orthodontic tooth movement in adults. World J. Orthod 2009; 10:141-6.

Yasuda H, Shima n, Nakagawa n, Mochizuki SI, Yano K, Fujise n, Sato Y, Goto M, Yamaguchi K, Kuriyama M, Kanno T, Murakami A, Tsuda E, Morinaga T, Higashio K. Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. Endocrinology 1998;139:1329-37.

Yildirim S, Yapar M, Sermet U, Sener K, Kubar A. The role of dental pulp cells in the resorption of deciduous teeth. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;105:113-20.

Zengo AN, Pawluk RJ, Bassett CAL. Stress-induced bioelectric potentials in the dentoalveolar complex. Am J Orthod 1973;64:17-27.

VITA

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